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Title of Dissertation: "Disruption of Inhibitory Function in the Ts65Dn Mouse Hippocampus Through Overexpression of GIRK2"

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Disruption of inhibitory function in the Ts65Dn mouse hippocampus through overexpression of GIRK2

by

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Doctoral Dissertation submitted to the faculty of the Graduate Program in Neuroscience of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 2007

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ABSTRACT

Disruption of inhibitory function in the Ts65Dn mouse hippocampus through overexpression of GIRK2

by

Tyler K. Best

Down syndrome (DS) is the most common nonheritable cause of mental retardation. DS is the result of the presence of an extra chromosome 21 and its phenotype may be a consequence of overexpressed genes from that chromosome. One such gene is Kcnj6/Girk2, which encodes the G-protein coupled inward rectifying potassium channel subunit 2 (GIRK2). The DS mouse model, Ts65Dn, overexpresses GIRK2 throughout the brain and in particular the hippocampus. With the advent of mouse models of Down syndrome (DS) the possibilities to further explore and understand the dysfunctions associated with DS expands. In particular, the basic and cellular neurophysiology can now be achieved in mice, which was not possible in human DS. Such that specific DS dysfunctions can now be separated from the overall disorder and examined in a manner providing interventions directly addressing particular abnormalities. The function of well defined regions and even certain genes within the triplicated chromosome are being understood with greater clarity to the extent that some DS specific phenotypes could be therapeutically ameliorated in the future. For example, the phenotype in a neurofibromatosis mouse model was reversed with pharmacological treatment.

Here, I report that the overexpression of GIRK2 leads to a significant increase (~2 fold) in $GABA_B$ mediated GIRK current in primary cultured hippocampal neurons. The dose response curves for peak and steady state GIRK current density are significantly shifted left towards lower concentrations of baclofen in Ts65Dn neurons compared to diploid controls, consistent with increased functional expression of GIRK channels. Stationary fluctuation analysis of baclofen-induced GIRK current from Ts65Dn neurons indicated no significant change in single channel conductance compared to diploid. However, significant increases in GIRK channel density were found in Ts65Dn neurons. In normalized baclofen-induced GIRK current and GIRK current kinetics no difference

was found between diploid and Ts65Dn neurons, which suggest unimpaired mechanisms of interaction between GIRK channel and $GABA_B$ receptor.

Since GABAergic dysfunction in Ts65Dn has been implicated in synaptic plasticity and learning and memory deficits, we sought to evaluate basal synaptic GABAergic transmission in CA1 pyramidal neurons. Ts65Dn mice showed an ~3 fold significant increase in spontaneous inhibitory postsynaptic currents (IPSC) frequency that was not matched by an increase in miniature IPSC frequency suggesting hyperexcitable interneurons. Hippocampal CA1 pyramidal neurons receive stratum specific direct (temporoammonic-TA) and indirect (trisynaptic, Schaffer collateral-SC) cortical inputs. TA fibers terminate at the most distal dendrites within the stratum lacunosum-moleculare, whereas, SC terminals are restricted to the more proximal stratum radiatum. Synaptic $GABA_B$/GIRK currents evoked by stimulation at TA zone of Ts65Dn CA1 neurons are significantly elevated and their frequency-dependence is impaired. Synaptic $GABA_A$ currents evoked by stimulation at TA zone appear to be normal although their frequency dependence is defective suggesting abnormalities in synaptic $GABA_A$ system as well. None of the properties of synaptic $GABA_B$ and $GABA_A$ mediated currents evoked by stimulation of SR appear to be affected in Ts65Dn CA1 neurons. These results indicate that GIRK2 overexpression in Ts65Dn has functional consequences which affect the balance between $GABA_B$ and $GABA_A$ inhibitory transmission in an input specific manner. We propose that cortical-hippocampal circuitry involving inhibitory TA terminals can contribute to cognitive dysfunction in DS individuals. The temporoammonic pathway (SLM) from entorhinal cortex to the hippocampus is a major route of information transfer underlying memory consolidation. Alterations in inhibitory modulation at these terminals can explain cognitive dysfunction in DS individuals.

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Katrina—whom I love

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Myron—for his quiet concern

Vivian—for her righteous weavings

My Advisor

Zygmunt—for his propellant enthusiasm

LIST OF ABBREVIATIONS:

AMPA:	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AHP:	after-hyperpolarization
AP:	action potential
App:	amyloid precursor protein
CA1:	cornu ammonis field 1
CA2:	cornu ammonis field 2
CA3:	cornu ammonis field 3
CaMKII:	Ca^{2+}/calmodulin-dependent protein kinase II
CGP55845:	specific $GABA_B$ receptor antagonist
CNS:	central nervous system
CNQX:	kainate/AMPA glutamate receptor antagonist
D-APV:	specific NMDA receptor antagonist
DS:	Down syndrome
DSCR:	Down syndrome critical region
EPSC:	excitatory post-synaptic current
EPSP:	excitatory post-synaptic potential
GABA:	gamma-aminobutyric acid
GIRK:	G-protein coupled inward rectifying potassium channel
GPCR:	G-protein coupled receptor
Glu_{K5}:	glutamate receptor subunit-5 kainate subtype
Hsa21:	human chromosome 21
Kir:	potassium inward rectifier
IPSC:	inhibitory post-synaptic current
mIPSC:	miniature inhibitory post-synaptic current
IPSP:	inhibitory post-synaptic potential
LTD:	long-term depression
LTP:	long-term potentiation
mEPSC:	miniature excitatory post-synaptic current
mIPSC:	miniature inhibitory post-synaptic current
Mx1:	myxovirus (influenza virus) resistance-1
NMDA:	N-methyl-D-aspartate
OAI:	stratum oriens/alveus interneuron
PIP_2:	phosphatidyl inositol bisphosphate
PKC:	protein kinase C
PLC:	phospholipase C
PPF:	paired pulse facilitation
RGS:	regulators of G-protein signaling
SC:	Schaffer collateral
SLM:	stratum lacunosum-moleculare
SO:	stratum oriens
Sod1:	superoxide dismutase-1
SR:	stratum radiatum
TA:	temporoammonic
TBS:	theta burst stimulation
Tiam1:	T-lymphoma invasion and metastasis-1
ZD7288:	specific antagonist of after-hyperpolarization currents (I_H)

CHAPTER 1

INTRODUCTION

In 1866 the English physician, John Langdon Down, first illustrated the characteristic features of individuals which we now associate with trisomy 21 or Down syndrome (DS) (Down, 1866). The incidence and extent of phenotypes varies with each individual. However, all DS individuals have mental retardation, muscle hypotonia and later in life, show Alzheimer disease-like pathology (Antonarakis et al., 2004). Other phenotypes include craniofacial deformities, shortened stature, congenital heart defects and in a minority of DS individuals, acute megakaryocytic leukemia (Epstein, 2002; Roizen and Patterson, 2003). In 1959, the French geneticist, Jérôme Lejeune, showed that DS is caused by chromosomal abnormalities related to the presence of an extra whole or part of chromosome 21, termed trisomy 21 (Lejeune, 1959).

There are more than 300 known genes encoded by human chromosome 21 (Hsa.21) (Hattori et al., 2000; Gardiner et al., 2003), which can contribute to the numerous DS phenotypic abnormalities. A chromosomal Down syndrome critical region (DSCR) for many of the neurological features such as mental retardation has been hypothesized to be localized between the carbonyl reductase (*CBR*) and transcriptional regulator *ets*-related gene (ERG) loci (Delabar et al., 1993; Dahmane et al., 1995; Toyoda et al., 2002). However, genes outside the DSCR also can be involved in the DS phenotype (Korenberg et al., 1994) and recently the concept and existence of the DSCR has been challenged in a mouse model which did not demonstrate some of the typical DS phenotypes (Olson et al., 2004).

Trisomy mouse models provide insight into the molecular and genetic effects that abnormal chromosome number has upon neurophysiological profiles. The distal segment

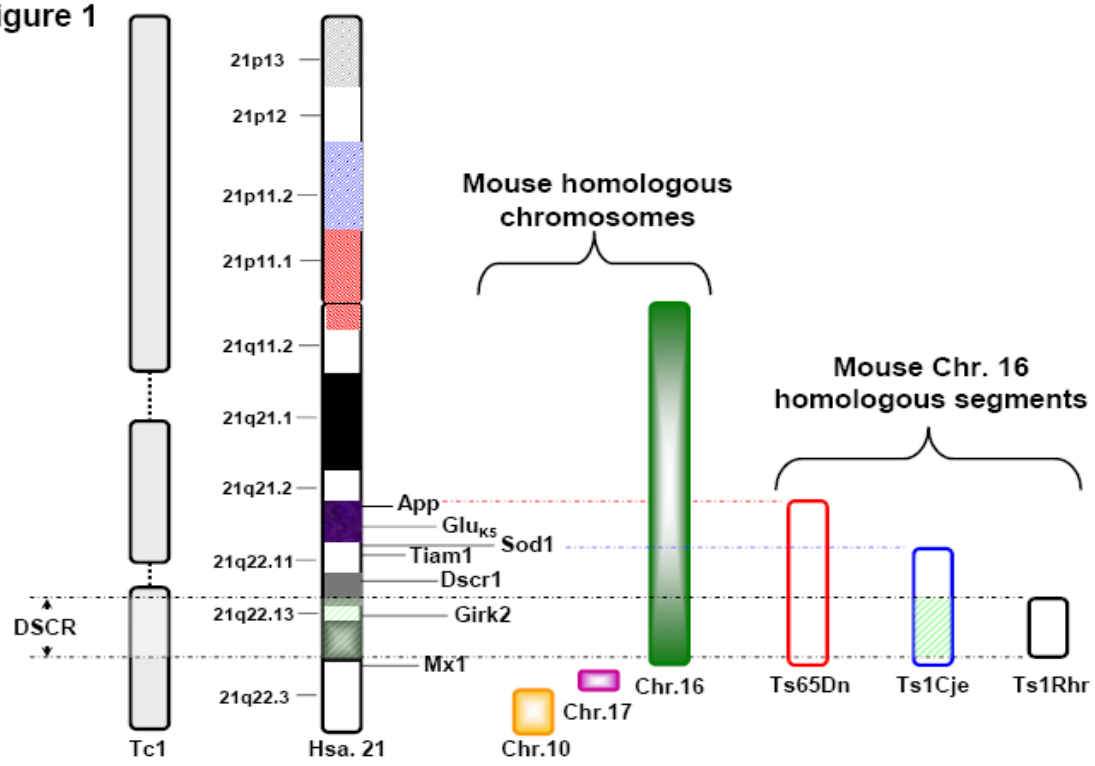

Figure 1. Human chromosome 21 (Hsa. 21) map showing the Down syndrome critical region (DSCR) and relationship of various genes to the trisomy mouse model chromosome segments. Homologous segments of mouse chromosomes 10, 16 and 17 are also represented corresponding to analogous portions of Hsa. 21.
Noted Genes: amyloid precursor protein (App), glutamate receptor subunit-5 kainate subtype (GluK5), superoxide dismutase-1 (Sod1), T-lymphoma invasion and metastasis-1(Tiam1), Down syndrome candidate region-1 (Dscr1), G-protein coupled inward rectifying potassium channel subunit-2 (Girk2), myxovirus (influenza virus) resistance-1 (Mx1).

of mouse chromosome 16 is homologous to nearly the entire long arm of Hsa.21 (Figure 1). Of the more than 300 genes of Hsa.21, 170 genes are highly conserved in mouse (Gardiner et al., 2003). Of these 170 mouse orthologs, ~66% are found on mouse chromosome 16, ~23% on mouse chromosome 10, and ~11% on mouse chromosome 17. Therefore, mice with full or segmental trisomy 16 (Ts65Dn, Ts16Cje and Ts1Rhr) are considered genetic animal models of DS. Ts65Dn and Ts1Cje mice demonstrate impaired

learning in spatial tests and abnormalities in hippocampal synaptic plasticity, which mirrors deficits seen in DS individuals. Mice in which only the DSCR is triplicated (Ts1Rhr) show no abnormalities related to craniofacial features or deficits in LTP (Olson et al., 2004; Olson et al., 2007). Recently an aneuploid mouse strain carrying ~92% of Hsa.21 has been developed (Tc1) (O'Doherty et al., 2005). Although chimeric (~66% of brain nuclei were positive for Hsa.21) for Hsa.21, these mice show many phenotypes consistent with DS and the various DS mouse models, such as spatial learning and memory deficits, abnormal synaptic plasticity, decrease in cerebellar granule cell populations, developmental heart problems and decreased mandibular size. In all, these newly created trisomic mouse models provide valuable tools by which to elucidate dysfunctions found in DS individuals.

Many explanations as to why trisomy 21 results in DS phenotypes have been postulated (Antonarakis and Epstein, 2006). The first possibility is augmentation of functional aspects of triplicated gene products contribute to the DS phenotype. That is to say, when a gene is overexpressed, the function of that gene is also increased. This may involve a few genes with major functional contributions or many genes with moderate functional contributions. This includes the possibility that triplicated gene products functionally interact with non-triplicated gene products with the consequence that may be an increase in function of non-triploid genes. A second explanation is also related to gene dosage but is more qualitative rather than a merely quantitative effect of trisomy 21. The idea is that an increased amount of gene product, regardless of the function of that product, overwhelms cellular and systemic functions. The increased content ‘gums up the works’ so to speak. The third explanation why trisomy 21 results in Down syndrome is

that functional non-protein-coding DNA elements from the triplicated region are involved. Non-coding DNA sequences such as *cis* and *trans* elements or RNAi when in excess disrupt the balance of gene transcription and translation (Antonarakis and Epstein, 2006). All three explanations may contribute to DS phenotype and it is likely a combination thereof which causes DS. An important point to consider with triploid genes, is that genes from the third copy of Hsa.21 may strictly follow gene dosage in that they are overexpressed by ~50%. However, data indicate not all triploid genes follow gene dosage (Chrast et al., 2000). This is likely due to genetic interactions such as changes in transcription factor expression that can subsequently up- or down regulate other gene expression levels.

My thesis work has focused on evaluating the first explanation, i.e. that specific genes within the triplicated chromosome contribute to DS; in particular, the overexpression of GIRK2 and the functional consequences of its overexpression. *Kcnj6 (Girk2)* encodes subunit 2 of the G-protein inward rectifying potassium channel (GIRK2) and is located within the so-called DSCR. It is the only ion channel subunit encoding gene within the DSCR that is expressed at high levels within the CNS and in particular within hippocampal and cerebellar tissues where DS deficits are prominent (Galdzicki and Siarey, 2003). We found that GIRK2 mRNA and protein subunits are highly overexpressed in multiple CNS structures including the hippocampus (Figure 2) (Harashima et al., 2006). The extra gene copy of *Girk2* and its overexpression can plausibly explain disruptions in neuronal homeostasis and synaptic function and contribute to cognitive dysfunction in DS. Recent work has shown that GIRK channels may indeed be involved in some DS deficits. Chronic treatment of Ts65Dn mice with a

partial GIRK channel antagonist (Kobayashi et al., 2003), increased neurogenesis of hippocampal neurons to levels comparable with diploid mice (Clark et al., 2006).

Figure 2

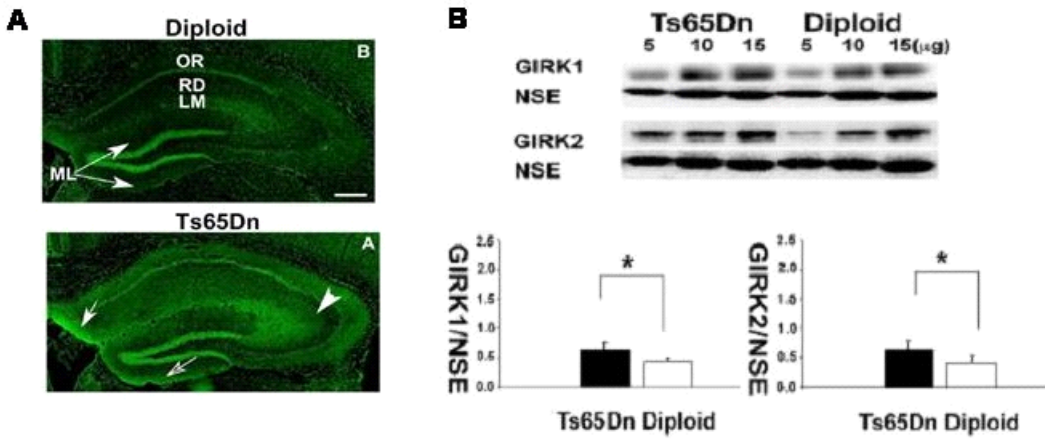

Figure 2. Increased expression of GIRK channel subunits in Ts65Dn hippocampus Images of GIRK2 immunoreactivity **(A)** in diploid (top) and Ts65Dn (bottom) hippocampus indicate increased immunoreactivity in Ts65Dn. Western blots **(B)** demonstrate significant increases in GIRK1 and GIRK2 expression in homogenates of Ts65Dn hippocampi compared to diploid (*p<0.05). Neuron specific enolase (NSE) used as standard. (Adapted from Harashima et. al. 2006).

DS Mouse Models

ABNORMAL SYNAPTIC PLASTICITY IN DS MOUSE MODELS

The hippocampus is part of the limbic system and plays an important role in learning and memory. It is a site for long-term synaptic plasticity that appears to be critical to memory formation, consolidation and retrieval and therefore has been extensively studied in the modeling of learning and memory. Brief high-frequency activation of specific inputs causes a persistent increase in synaptic responsiveness (an increase in the excitatory postsynaptic potentials termed long-term potentiation (LTP))

that under certain circumstances can last for hours, days or weeks (Andersen, 2007). Investigations of LTP in the CA1 region of hippocampi from Ts65Dn mice found there to be reduced LTP over a period of 60 min compared to that of age-matched diploid-controls (Figure 3 insets) (Siarey et al., 1997). No significant differences in short term

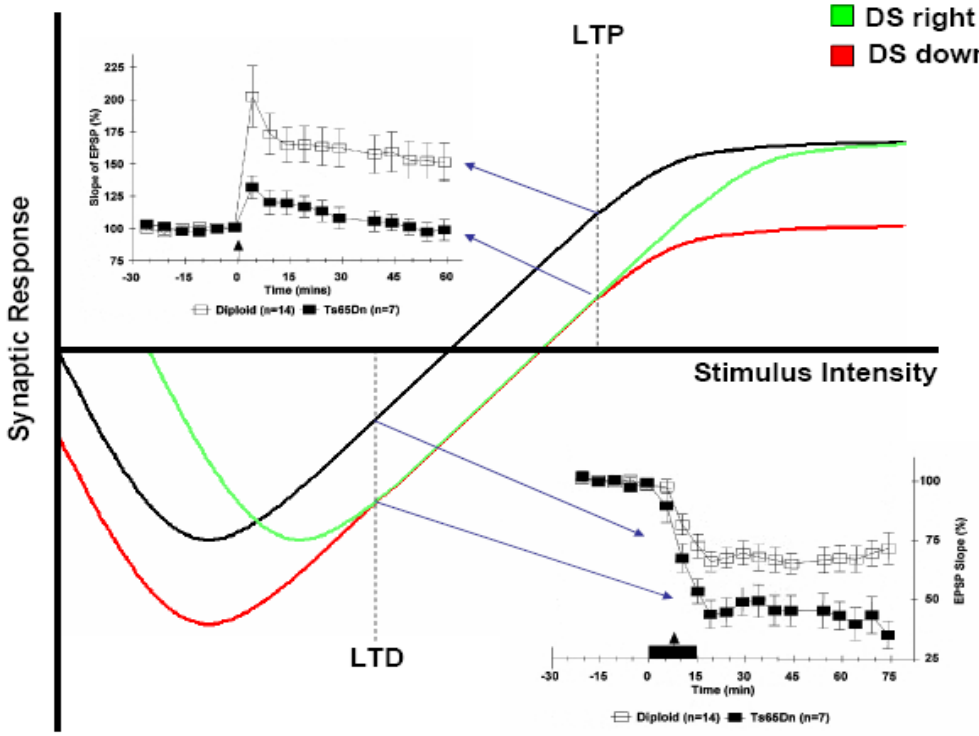

Figure 2. Possible shifts of the stimulus response curve for hippocampal synapses by trisomy mouse models of DS. Stimulus protocols that elicit LTP or LTD are represented by vertical dashed lines and corresponding points along the diploid (black), right (green) or down (red) shifted curves are represented by levels of potentiation or depression from representative responses of Siarey et al., (1999). The direction of shift is unknown (both right and downward shifts are possible with the known data) and further experiments are needed to determine saturation and relative levels of potentiation and depression.

plasticity between Ts65Dn and diploid controls were found in paired-pulse protocols, suggesting that presynaptic plastic mechanisms are similar. In another set of experiments sequential LTP and long-term depression (LTD) were evoked from Ts65Dn hippocampi (Siarey et al., 1999). Both forms of use-dependent synaptic plasticity were abnormal in

Ts65Dn compared to the diploid-controls; with LTP decreased, and LTD increased. Likewise Ts1Cje mice show decreased CA1 LTP and increased LTD (Siarey et al., 2005).

Recently LTP from the Tc1 mouse dentate gyrus was investigated and also found to be impaired, while baseline and short term plasticity were unchanged (O'Doherty et al., 2005). These data parallel findings from the other DS mouse models. Behavioral tests of Tc1 mice correlate with the synaptic plasticity data, in that short-term memory was intact in the alternating T-maze, but long-term memory was impaired as evidenced by the novel-object recognition task. In the mouse where only the DSCR region is triplicated, our laboratory recently reported that there are no significant differences in LTP between diploid and Ts1Rhr (Olson et al., 2007).

Mechanisms that explain the deficits in LTP from these mice are unclear; however, deficits in LTP of Ts65Dn hippocampus were described by an increase in GABAergic tone. Impairments in LTP from the dentate gyrus of the Ts65Dn mouse were not present when $GABA_A$ receptors were blocked by 100 μM picrotoxin (Kleschevnikov et al., 2004). Likewise Ts65Dn dentate neurons showed an increased frequency of miniature inhibitory postsynaptic currents (mIPSCs) and a decrease in paired-pulse facilitation (PPF) of evoked IPSCs, findings consistent with an increase in GABA release probability. In another study, 10 μM picrotoxin eliminated a deficit in theta-burst stimulus (TBS) induced LTP in Ts65Dn CA1 hippocampal region (Costa and Grybko, 2005). Furthermore, normal levels of LTP were elicited in Ts65Dn dentate gyrus upon chronic (2 weeks) oral administration of sub-seizure threshold doses of another $GABA_A$ antagonist, pentylenetetrazole, an effect that coincided with normal cognitive function in

an object discrimination test (Fernandez et al., 2007). These data suggest that an increased inhibitory tone may lead to diminished LTP of Ts65Dn hippocampus.

GENETIC CONTRIBUTIONS TO ABNORMAL CNS FUNCTION

The contribution of the many trisomic genes to the deficits in synaptic plasticity remain unclear, however the use of DS mouse models that contain different (e.g. smaller) trisomic segments can clarify the genes involved in the dysfunction. For example, Ts1Cje or Ts1Rhr mice, which contain smaller triplicated segments than the Ts65Dn mouse are employed (see Figure 1). Studies examining the behavior of Ts1Cje mice reveal cognitive and behavioral abnormalities including low levels of locomotion, decrease in exploratory behavior, and impairment in spatial learning in the hidden platform and reverse hidden platform tasks in the Morris water maze (Sago et al., 1998; Sago et al., 2000). The latter of these studies were performed on Ts65Dn and Ts1Cje littermates (derived from the same mating scheme, thus carrying identical genetic background) and therefore the degree of impairment can be directly compared. Comparisons with the Ts65Dn mouse show that, although the Ts1Cje mouse had similar spatial learning deficits, these deficits were less severe than those found in the Ts65Dn mouse (Sago et al., 1998; Sago et al., 2000). However, in contrast to Ts65Dn mice, Ts1Cje mice perform normally in short-term and long-term novel object recognition tasks (Fernandez and Garner, 2007; Fernandez et al., 2007).

Experiments of synaptic plasticity show similar chromosomal size effects. LTP and LTD in the isolated hippocampus of Ts1Cje mice are abnormal compared to diploid controls. LTP was reduced and LTD was augmented in comparison to diploid controls

(Siarey et al., 2005). These changes are significant, but are less dramatic than were seen in Ts65Dn mice under identical experimental protocols. Identical LTP protocols in Ts1Rhr mice however, did not demonstrate deficits in levels of potentiation (Olson et al., 2007). These findings suggest that the smaller chromosomal segment has a diminished functional impact on learning and behavioral tasks as well as on synaptic plasticity. Whether these differences are related to specific genes in the triplicated segments or to the quantity of genetic material remains to be examined.

Genes from within the common trisomic segment most likely cause the abnormal CNS function. One such gene could be *Girk2*. G-protein coupled inward rectifying potassium (GIRK) channels contribute to neuronal resting potential, excitability and firing properties. We have shown that GIRK2 protein is overexpressed ~1.5 fold in Ts65Dn hippocampus (Harashima et al., 2006) and is likely to be overexpressed in Ts1Cje as well. In GIRK2 knockout mice, LTP and LTD are also abnormal (Adeniji-Adele et. al., 2004) but in the opposite direction from the DS mouse models, suggesting that the level of GIRK2 expression correlates to the level of potentiation or depression of hippocampal synapses.

Given the limited dynamic range of Schaffer-collateral/CA1 synapses (Savicc et al., 2003), a shift in the baseline synaptic strength could explain both a decrease in LTP and an increase in LTD for the Ts65Dn and Ts1Cje mouse models (Figure 3). The expression level of GIRK2 could effectively change the dynamic range of hippocampal synapses. Multiple G-protein coupled receptors, including the metabotropic $GABA_B$ receptor activate GIRK currents. GIRK channels have been shown to be highly expressed and constitutively active in CA1 pyramidal cell dendrites (Chen and Johnston, 2005;

Koyrakh et al., 2005) and therefore likely to dramatically influence synaptic function when overexpressed. $GABA_B$-mediated slow IPSCs are sufficient to inhibit NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) in dentate molecular layer interneurons (Mott et al., 1999). These slow IPSCs also block action potentials evoked by weak but not strong depolarizations (Mott et al., 1999). Furthermore, overexpression and activation of GIRK channels in cultured rat hippocampal neurons resulted in hyperpolarization by 11-14 mV and depleted action potential (AP) firing by increasing AP threshold 2- to 3-fold (Ehrengruber et al., 1997).

Note: Much of this section discussing DS mouse models as well as Figures 1 and 3 were adapted from my review published online in Cell Science Reviews see Appendix and (Best et al., 2006).

GIRK Channels

GIRK CHANNEL STRUCTURE

GIRK channels are members of the large family of potassium inward rectifiers (Kir). The seven subfamilies of Kir channels (Kir1-7) differ as to the degree of their rectification and their regulation by intracellular signals. GIRK channels rectify strongly and are gated by G-protein signaling. To date four mammalian genes encoding GIRK subunits have been isolated which are designated Kir3.1 (*Girk1*), Kir3.2 (*Girk2*), Kir3.3 (*Girk3*) and Kir3.4 (*Girk4*) (Lesage et al., 1994; Isomoto et al., 1997). A fifth subunit, Kir3.5 (*Girk5*), has also been identified in xenopus oocytes (Hedin et al., 1996). GIRK

channels are composed of a tetrameric complex of alternating paired subunits that form a K^+ selective pore (Nishida and MacKinnon, 2002). Each subunit is composed of two transmembrane domains (M1 and M2) on either side of a pore loop which contributes to the K^+ selectivity filter of the pore (Bichet et al., 2003). Both C- and N-termini are intracellular and interactions with these domains by other intracellular molecules regulate channel gating.

The composition of GIRK channel assembly is dependent on tissue and cell type. In cardiac muscle GIRK channels are generally formed of GIRK1 and GIRK4 subunits while in the CNS GIRK channels are primarily composed of GIRK1 and GIRK2 subunits (Thomas et al., 1997; Wischmeyer et al., 1997). In all studies of rat and mouse, GIRK4 expression throughout brain regions is minimal or undetected compared to other GIRK channel subunits (however, see (Iizuka et al., 1997; Wickman et al., 2000). Homomeric channels are generally less functional than are heteromeric complexes of GIRK1 and at least one of the other GIRK subunits (Kofuji et al., 1995; Ehrengruber et al., 1997; Wischmeyer et al., 1997; Corey and Clapham, 1998; Corey et al., 1998; Schoots et al., 1999). There are certain tissues where only homomeric channels are formed. Atrial muscle express homotetrameric GIRK4 channels, while substantia nigra dopaminergic neurons express homotetramers of GIRK2 splice variants (Corey and Clapham, 1998; Inanobe et al., 1999). Trafficking and assembly of GIRK channels in the CNS and heart is dependent on GIRK2 and GIRK4 subunits respectively while GIRK3 expression inhibits plasma membrane expression and targets the channel assembly for lysosomal degradation (Ma et al., 2002). The N-terminal domains in the GIRK2 and GIRK4 subunits contain endoplasmic reticulum export motifs and the C-terminal domains

contain surface promoting motifs that transfers channels from endosomes to the cell surface (Ma et al., 2002). Interestingly, in *Girk2* knockout animals there are consequential decreases in GIRK1 protein expression and conversely with *Girk1* knockouts, there is also a decrease in GIRK2 protein expression (Marker et al., 2004; Koyrakh et al., 2005). Decreased expression of GIRK1 and GIRK2 subunits were not found in *Girk3* knockouts. This correlates well with results from our lab in which GIRK2 overexpression in Ts65Dn mice drives overexpression of GIRK1 subunit but not mRNA of *Girk1* (Harashima et al., 2006). Trafficking of GIRK channels to membrane domains has also been shown to be regulated by N-methyl-D-aspartate receptor (NMDA-R and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) signaling involving protocols that induce AMPA receptor LTP (Huang et al., 2005). These finding indicate that GIRK channel formation and trafficking to functional domains is a dynamic and highly regulated process.

Like all potassium channels, Kir channels can discriminate between K^+ and Na^+. This ability to discriminate against the smaller Na^+ (atomic radius: 0.95 Å) was elucidated by examining the pore structure of the bacterial KcsA channel (Roux and MacKinnon, 1999; Morais-Cabral et al., 2001; Zhou et al., 2001; Choe, 2002). All solved crystal structures indicate that K^+ selectivity is due to main chain carbonyl oxygen atoms of the conserved signature sequence TXGYG or TXGFG (Bichet et al., 2003). Point mutations in this signature sequence abolish K^+ selectivity and account for the movement disorder in *Weaver* mice (Heginbotham et al., 1994; Patil et al., 1995). Selectivity for K^+ is based on the energetically favorable environment provided by the main chain carbonyl oxygen atoms which form a series of four K^+ binding sites within the pore. At the

extracellular and intracellular surface, K^+ ions are encompassed in a hydrated shell. As K^+ ions enter the filter the surrounding water molecules are progressively shed and replaced by interactions with the oxygen atoms of the filter. Dehydrated K^+ ions occupy two of the four a binding sites and move sequentially through the pore (site 1 and 3 then site 2 and 4). The pore region is rigid and the inter-atomic distances are such that the smaller Na^+ is not supported by the oxygen atoms of the filter.

MECHANISMS OF INWARD RECTIFICATION

Inward rectification refers to the ability of a channel to allow greater influx than efflux of ions. Intracellular cations like Mg^{2+} and polyamines such as spermine, block GIRK conductance at depolarized membrane potentials and give the channel inward rectifying characteristics. The intracellular domains of Kir channels extend the ion pore nearly twice as long as other K^+ channels. This extended cytoplasmic pore is lined with a large number of polar amino acids, many of which are negatively charged (i.e. Glu and Asp), providing a relatively negative electrostatic potential which promotes K^+ concentration and thus facilitating conductance. The pore characteristics also create favorable conditions for impermeant cations and polyamines to block the pore from the inside (Nishida and MacKinnon, 2002). Mg^{2+} mediated block is thought to depend primarily upon charged amino acids of the second transmembrane segment. Mutation to a negatively charged residue (N171D or N171E) within this domain localized along the pore of the central cavity changes the affinity for Mg^{2+} and can convert the Kir from a weak rectifier like the ROMK1 channel to a strong rectifier like GIRK1 (Lu and MacKinnon, 1994). High affinity interactions between polyamines and the lining of this

extended pore region also promote rectification of Kir channels. The negatively charged residues of the pore lining are interspersed with hydrophobic side chains. This arrangement provides a complementary match for certain polyamines to bind and block Kir channels, such as spermine for GIRK (Nishida and MacKinnon, 2002).

Because of rectification, at depolarized membrane potentials GIRK channels are less conductive and less capable of shunting excitatory currents. Conversely at resting membrane potentials GIRK channels are conductive and are capable shunts. Sufficient depolarization by excitatory inputs would limit the extent of GIRK channel conductance and thus allow additional excitatory currents to elicit greater responses. In contrast, relatively small excitatory currents that are not sufficient to depolarize the membrane to a range where GIRK conductance rectifies would not be able to facilitate additional excitatory inputs to further depolarize the membrane. The expression levels and subcellular localization of GIRK channels, as well as the relative activity at G-protein coupled receptors thus serve as a mechanism by which neurons utilize GIRK channels to gate excitatory inputs. Just as NMDA receptors serve as coincidence detectors of depolarization and glutamatergic signaling in synaptic plasticity (Bliss and Collingridge, 1993), GIRK channels may serve as coincidence detectors of excitatory inputs by regulating the integration of signals in the dendritic arbor and thus can regulate whether or not membrane currents sufficiently depolarize the membrane to reach firing threshold at the soma.

GIRK CHANNEL ACTIVATION

The mechanisms for modulating GIRK channels have been studied intensively and have been shown to involve a variety of factors, such as Gβγ, Gα, intracellular Na^+ and phosphatidyl inositol bisphosphate (PIP_2) (Mark and Herlitze, 2000; Kovoor and Lester, 2002; Sadja et al., 2003). Not one factor independently gates channel opening, but all seem to act in concert to regulate GIRK activity. Gβγ subunits were first demonstrated to activate potassium conductance by Logothetis et al., (1987). Upon agonist application at Gi/o coupled receptors, the Gβγ subunits act on cytosolic domains of GIRK channel subunits causing channel opening. Gα has also been shown to act directly with the N–terminus of channel subunits to reduce the basal current (Peleg et al., 2002). Intracellular Na^+ concentration influences GIRK channel current by neutralizing negatively charged residues on the C-termini of GIRK2 and GIRK4 channel subunits allowing PIP_2 to interact with and activate the channel (Ho and Murrell-Lagnado, 1999a, 1999b). PIP_2 interactions with GIRK channels are a necessary element involved in activating current in these channels. Free Gβγ subunits have been shown to stabilize the channel-PIP_2 interactions (Ho and Murrell-Lagnado, 1999a) and synthesis of PIP_2 by lipid kinases activates GIRK channels in cardiac myocytes (Hilgemann and Ball, 1996). In oocytes heterologously expressing channels composed of GIRK1 and GIRK4 subunits or solely GIRK2 subunits, the addition of Gβγ subunits alone did not activate GIRK current after rundown. However, PIP_2 activated GIRK current within minutes when applied alone and within seconds when applied in conjunction with Gβγ (Huang et al., 1998). This suggests that Gβγ subunits are not sufficient to activate GIRK current alone, however, PIP_2 is. Furthermore, rundown of GIRK current is increased when PIP_2 levels

are diminished through activation of PLC or through inhibition of the phosphoinositol kinases that generate PIP_2 (Cho et al., 2001). Additionally, when exposed to PIP_2 antibody, current is abolished in GIRK1/4 and GIRK2 channels (Huang et al., 1998).

Multiple neurotransmitters are known to modulate GIRK activity. For the most part, any neurotransmitter that activates a Gi/o coupled receptor can activate GIRK channels when in proximity to each other. Whereas, those acting at Gq coupled receptors tend to inhibit GIRK channels either through hydrolysis of PIP_2 or PKC mediated phosphorylation of GIRK subunits, or both (Sharon et al., 1997; Mao et al., 2004). Indeed, in native neurons and expression systems, acetylcholine, adenosine, endocannabinoids, dopamine, GABA, glutamate, histamine, melatonin, neuropeptide Y, norepinephrine, orexins (hypocretins), opioids, serotonin, and somatostatin have all been shown to modulate GIRK channel activity (Kofuji et al., 1995; Kobayashi et al., 1996; Nelson et al., 1996; Saugstad et al., 1996; Spauschus et al., 1996; Kreienkamp et al., 1997; Luscher et al., 1997; Kuzhikandathil et al., 1998; McAllister et al., 1999; Takigawa and Alzheimer, 1999; Ulens et al., 1999; Bunemann et al., 2001; Hoang et al., 2003; Paredes et al., 2003). When active, GIRK channels conduct potassium ions at a relatively moderate rate, an estimated of 5-6 pS at physiological ionic concentrations but range from 20-30 pS under experimental high extracellular K^+ concentrations (Takigawa and Alzheimer, 1999; Chen and Johnston, 2005; Best et al., 2007).

Upon agonist application and removal, GIRK channel response displays characteristic activation, desensitization and deactivation periods. The kinetics of GIRK channel activation vary according to agonist concentration and G-protein subtype (Benians et al., 2003; Zhang et al., 2004). The varied kinetics are also dependent on the

intrinsic differences in receptor-catalyzed GDP release which limits the rate of Gβγ binding to and activating GIRK channels (Zhang et al., 2004). This is consistent with data showing that RGS proteins (Regulators of G-protein signaling), which regulate the GTP hydrolysis by Gα subunits, also regulate GIRK activation kinetics (Benians et al., 2003). GIRK activation kinetics are presumably not rate-limited by the frequency of random collisions with Gβγ since signaling components are likely compartmentalized or preassembled (Lober et al., 2006).

As is the case for activation kinetics, rapid desensitization (seconds) is dependent on G-protein cycling, concentration of agonist and on the classification of neurotransmitter receptor (Leaney et al., 2004). Long-term desensitization (minutes) however, involves a variety of factors that appear to be independent of the receptor classification (Sickmann and Alzheimer, 2003). Instead, G-protein cycling, phosphorylation of GIRK channels and receptor, as well as internalization of receptor have all been implicated in slow desensitization of GIRK currents (Kovoor et al., 1995; Shui et al., 1995; Bunemann et al., 1999; Sickmann and Alzheimer, 2003). Scavenging of Gβγ has also been suggested to contribute to slow desensitization (Blanchet and Luscher, 2002). The rate of PIP_2 hydrolysis is likely not a factor in slow desensitization but does lead to slow inhibition (Meyer et al., 2001; Cho et al., 2002; Sickmann and Alzheimer, 2003).

The rate-limiting factors involved in deactivation kinetics are dependent upon a combination of factors involving agonist and GPCR and G-protein cycling. In some cases, as in $GABA_B$ or muscarinic M_4 receptors, the deactivation rate is determined by GTP hydrolysis by Gα, as RGS proteins can accelerate deactivation kinetics; and since

various Gα subtypes show different deactivation rates (Benians et al., 2003). In contrast deactivation at the adenosine receptor A_1 was insensitive to RGS activity or Gα subtypes but was sensitive to the chemical nature of the agonist (Benians et al., 2003). This suggested that in this class of receptor, deactivation is determined by removal of agonist from the receptor.

Hippocampal neurons are likely to be exposed to multiple neurotransmitters such as serotonin, GABA, glutamate, somatostatin, adenosine and acetylcholine. All of which have been shown to activate inwardly rectifying potassium currents (Sodickson and Bean, 1998; Seeger and Alzheimer, 2001; Leaney, 2003). Since GIRK channels are common downstream targets of these neurotransmitters, it is important to understand how simultaneous activity of these various neurotransmitters affect GIRK channel activity. Are there microdomains within neurons where each GPCR has its own set of effector GIRK channels or do they share GIRK channels and G-proteins? What is the limiting factor in maximizing GIRK current? Is it the number of receptors, available G-proteins or GIRK channels that restrict the amount of potassium current? Serotonin and baclofen applied simultaneously in rat hippocampal slices elicited current no greater than that of baclofen alone (Andrade et al., 1986). Likewise, acutely dissociated hippocampal pyramidal neurons, when exposed to saturating concentrations of agonists at somatostatin, adenosine, and serotonin receptors also showed occlusive or sub-additive currents when applied with saturating concentrations of baclofen (Sodickson and Bean, 1998). This occlusive effect was not observed when sub-saturating agonist concentrations (~1/3 EC_{50}) were used. In fact, currents were supra-additive when sub-saturating concentrations of agonists were applied simultaneously, that is, current amplitude was

greater than when current amplitudes were summed from individual agonist applications (Sodickson and Bean, 1998). These results suggest that each receptor signals onto common pools of G-proteins and GIRK channels; and when saturated at one receptor another agonist cannot elicit more GIRK current because the pool is already activated. Conversely, when sub-saturating agonist concentrations are used and the pool of G-proteins is activated by the different receptors, there is cooperativity in activating GIRK channels and supra-additive current is observed. This may indicate that the GIRK channels are the limiting factor in the amplitude of current observed. An increase in GIRK expression (as in Ts65Dn) therefore, would shift the saturating threshold for simultaneous neurotransmitter occlusion to higher agonist concentrations and thus disrupt the precision of neurotransmitter effects.

SUBCELLULAR AND CNS LOCALIZATION OF GIRK CHANNEL

Plasma membrane associated GIRK1 and GIRK2 channel subunits have been identified primarily in extrasynaptic and perisynaptic regions of the postsynaptic membrane of mouse stratum radiatum (SR). GIRK2 but not GIRK1 was also found within postsynaptic specializations (Koyrakh et al., 2005). This suggests that within the SR, the less functional homomeric GIRK2 (or heteromeric GIRK2-3) channels exists at synaptic sites and the more functional heteromeric GIRK1-2 channels are found outside synaptic terminals. Dendritic shafts also showed immunoreactivity to GIRK2 when opposed by symmetric (putative GABAergic) terminals in both the SR and stratum lacunosum-moleculare (SLM) (Kulik et al., 2006). This shaft localization, however, was not associated with $GABA_{B1}$ suggesting that the receptor and channel are unlikely to be

coupled in dendritic shafts. In contrast, GIRK2 immunoreactivity colocalized with $GABA_{B1}$ on dendritic spines preferentially localized around asymmetric synapses (Kulik et al., 2006). This colocalization of both $GABA_{B1}$ and GIRK suggests functional coupling at perisynaptic regions of glutamatergic terminals. Shunting of excitatory currents may therefore be maximized by this localization. Interestingly, in rat hippocampus, extrasynaptic $GABA_B$ receptors show heterogenous potassium currents and may not couple to Ba^{2+}-sensitive inward rectifying potassium channels, whereas synaptic $GABA_B$ receptors are homogenous and solely elicit characteristic GIRK currents (Pham et al., 1998).

Hippocampus

High hippocampal GIRK expression indicates the importance of GIRK channels in modulating learning and memory. Indeed reports have established that rodents lacking GIRK subunits demonstrate defective learning and memory. GIRK4 knockout mice exhibited impaired performance in the Morris water maze (Wickman et al., 2000) and GIRK2 knockout mice demonstrated reduced ethanol-induced conditioned taste aversion and conditioned place preference (Hill et al., 2003). GIRK1 knockdown in rat impairs learning and memory in olfactory associative tasks (Kourrich et al., 2003).

Transcripts from *Girk1*, *Girk2* and *Girk3* have been identified throughout the hippocampus and reflect its laminar structure (Karschin et al., 1996; Chen et al., 1997). Most mRNA labeling however, was detected in pyramidal layers of CA1-CA3 and within the granular cell layer of dentate gyrus (Dissmann et al., 1996; Karschin et al., 1996; Chen et al., 1997). GIRK2 protein expression is very high in both cell body and fibers of

dentate, CA1-CA3, tenia tecta and indusium grisseum (Murer et al., 1997). GIRK1 immunoreactivity is predominantly found in dendritic arbors, spines and cell somata within CA1 of adult rat brain (Bausch, 1998). Although GIRK1 immunoreactivity appeared in strata of the dentate gyrus and CA3, the most intense signal was at the SLM within CA1. The intensity of GIRK1 immunostaining within CA1 dissipated outward from SLM to stratum oriens (SO). Within CA3 the labeling is similar to CA1 except that the strongest signal was found in the outer SO. Dentate gyrus GIRK1 immunostaining was the strongest at the outer stratum-molecular and decreased inward to be minimal within the hilus (Drake et al., 1997). In a second study, GIRK1 immunoreactivity for the

Figure 4

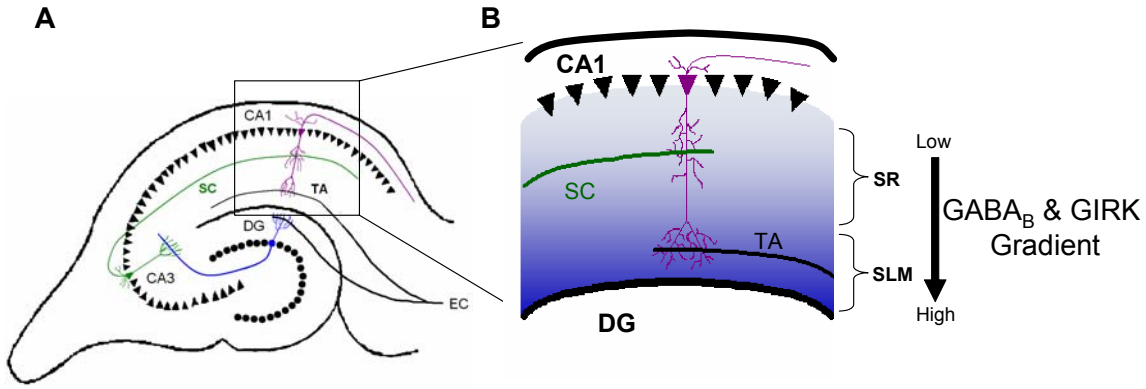

Figure 4. Hippocampal circuitry diagram with GABA$_B$ and GIRK expression gradient within CA1
Basic hippocampal circuitry **(A)** including entorhinal cortex (EC) projections to the dentate gyrus (DG; perforant path--indirect trisynaptic circuit), and distal dendrites (stratum lacunosum-moleculare--SLM) of CA1 pyramidal neurons (temporoammonic pathway--TA). The trisynaptic circuit continues with DG granule cell axons (mossy fibers) terminating on CA3 pyramidal neuron dendrites. CA3 pyramidal neuron axons project to the proximal dendrites (stratum radiatum--SR) of CA1 pyramidal neurons (Schaffer collaterals--SC) completing the trisynaptic circuit. CA1 pyramidal neurons are the primary output of the hippocampus. An expanded region (box) of CA1 **(B)** depicting the relative GABA$_B$ receptor and GIRK channel expression gradient within the CA1 region of the rodent hippocampus based on immunostaining from the following: GABA$_{B1}$R--Sloviter et. al., (1999); GABA$_{B2}$R--López-Bendito et. al., (2004); GIRK1--Drake et. al., (1997); GIRK2--Liao et. al., (1996). The gradient within the CA1 region presumably reflects a postsynaptic functional response of GABA$_B$ induced GIRK current gradient along CA1 pyramidal neuron dendrites, where low functional expression of GABA$_B$/GIRK responses is expected within the SR and high functional expression GABA$_B$/GIRK responses is expected in the SLM.

CA1, CA2 & CA3 areas was the most pronounced at the SLM and moderate throughout the remaining layers (Liao et al., 1996). GIRK2 detection, for the most part, followed this

pattern except in the pyramidal layer of CA2, where GIRK2 was relatively higher than SO or SR (Liao et al., 1996). Graded or regional expression of GIRK may be specific to certain populations of interneurons or may reflect differential functional expression of GIRK within glutamatergic neurons according to the type of connections they make with neighboring interneurons and other pyramidal neurons (Figure 4).

PHYSIOLOGICAL IMPACT OF GIRK CHANNEL ACTIVITY

Weaver mutant mice substantiate the importance of GIRK channels in proper neuronal functioning. These mice have a mutation in the pore loop selectivity filter of GIRK2 subunits which allows cations other than K^+, such as Na^+, to pass (Tong et al., 1996). This mutation results in mice which display ataxia, mild locomotor hyperactivity and occasionally, tonic-clonic seizures (Harkins and Fox, 2002). Neuron cell death occurs throughout the brain and especially the cerebellum as a result of this mutation. GIRK null mutants also have profound pathology. They show hyperactivity, increased incidence of seizure, and decreased analgesia (Signorini et al., 1997; Blednov et al., 2001; Blednov et al., 2002; Blednov et al., 2003; Marker et al., 2004). The phenotypic deficits of *Weaver* mice and GIRK channel subunit null-mutants indicate that proper GIRK channel conductance is vital to CNS function.

Potassium conductance, and in particular GIRK channel expression, correlates with neuronal resting potential, firing threshold and rhythmicity. Hippocampal neurons from GIRK2 subunit knockout mice show depolarized resting membrane potentials (Luscher et al., 1997; Koyrakh et al., 2005). Conversely, where GIRK channel is overexpressed, as in Ts65Dn or driven by adenoviral constructs, hippocampal neurons

are hyperpolarized (Table 2, Chapter 3; (Ehrengruber et al., 1997). Both the threshold current for action potential firing and spike frequency was unaffected with adenovirally driven overexpression of GIRK or in Ts65Dn neurons (supplemental Table 1, Chapter 3; (Ehrengruber et al., 1997). However, when GIRK agonists were applied, repetitive firing ceased and firing threshold was increased 2-3 fold in adenoviral GIRK channel overexpressing cells (Ehrengruber et al., 1997) (It should be noted that in this adenoviral overexpression system, GIRK channel number is not controlled, whereas in Ts65Dn there is exactly three *Girk2* gene copies).

Takigawa and Alzheimer (2003) elegantly showed that GIRK activation is sufficient to alter the computational properties of hippocampal neurons. GIRK channel activation altered the amplitudes and afterhyperpolarization of evoked excitatory post-synaptic potentials (EPSP). Synaptically evoked $GABA_B$ mediated responses (presumably through GIRK activity) in the SLM, where GIRK expression is relatively high within the hippocampus, have been shown to block CA1 pyramidal spikes driven by Schaffer collateral (SC) input (Dvorak-Carbone and Schuman, 1999). The efficacy of spike blocking matches the time course of $GABA_B$/GIRK mediated currents and shows a decline in efficacy with repeated SLM bursts consistent with desensitization of GIRK currents. Interestingly, the relative metaplastic state of synapses in the SLM demonstrated variable efficacy in blocking SC driven spikes. When synapses in the SLM were potentiated by an LTP inducing protocol, the ability to block SC driven spikes was enhanced, likewise LTD protocols in the SLM decreased the efficacy to block spikes (Remondes and Schuman, 2002). Furthermore, stimuli within the SLM was also able to impede the potentiation of SC-CA1 synapses by LTP protocols (Remondes and Schuman,

2002). These data indicate that $GABA_B$/GIRK activity in the SLM regulates the input and response of CA1 pyramidal neurons to SC stimuli.

SUMMARY

The mouse models of Down Syndrome (DS) provide a powerful tool to elucidate the mechanisms underlying suboptimal neural functioning in Down syndrome individuals at the neuronal level, and then affecting simple and complex neuronal networks. We can hypothesize that overexpression of genes from chromosome 21 shifts biological homeostasis in the Down syndrome brain to a new, less functional state. In this altered steady state mechanisms of development, structure, and plasticity malfunction due to compromises caused by the neurophysiological impact of overexpressed genes from trisomy 21. In particular, **I hypothesize that the three copies of the *Girk2* gene in DS drive functional overexpression of GIRK channels which results in dysfunctional inhibitory transmission within the Ts65Dn mouse hippocampus**. In order to evaluate this hypothesis I have experimentally evaluated the following aims:

Specific Aim #1: To determine whether GIRK current in hippocampal neurons is dependent on gene dosage in the Ts65Dn DS mouse model.

Dose-response curves to GIRK channel agonists can be utilized to quantify and compare GIRK currents between primary cultured hippocampal diploid and Ts65Dn neurons. Whole-cell voltage-clamp recordings were compared between genotypes and evaluated for basal and neurotransmitter induced GIRK channel currents. Coupling mechanisms between $GABA_B$ receptor, G-protein and GIRK channel were evaluated through analysis

of the kinetics of GIRK channel activation and deactivation kinetics. The results demonstrate increased $GABA_B$ mediated GIRK currents from Ts65Dn hippocampal neurons consistent with overexpressed GIRK channels. There was no evident disruption in coupling mechanisms indicating that increased GIRK currents are not associated with coupling variations.

Specific Aim #2: To establish the contribution of GIRK2 containing channel activity to inhibitory GABAergic post-synaptic currents (IPSCs) in the Ts65Dn DS mouse model.

GABA release at synapses acts on $GABA_B$ and $GABA_A$ receptors initiating slow and fast IPSCs respectively. The slow IPSCs are mediated through postsynaptic $GABA_B$ receptor activation of GIRK channels. If GIRK channel expression is functionally overexpressed then synaptic GABA release should induce slow IPSCs of greater magnitude. GABAergic synaptic transmission onto CA1 pyramidal neurons of hippocampal slices were elicited and evaluated for differences in the relationship between fast and slow IPSCs. Analysis of IPSCs showed significant increases in $GABA_B$/GIRK synaptic transmission.

Specific Aim #3: To evaluate the impact that GIRK2 containing channels has on signal integration and hippocampal circuitry in the Ts65Dn DS mouse model.

GIRK channels can shunt excitatory inputs and disrupt the timing of network signaling. Hippocampal inhibition regulates rhythmic oscillations and controls hippocampal output. GABAergic synaptic transmission was elicited from hippocampal slices for inputs at

proximal and distal CA1 dendrites. Short term plasticity of $GABA_A$ergic signaling, and integration of both $GABA_A$ and $GABA_B$ synaptic transmission were evaluated. This allows comparison between direct and indirect cortical inputs—the temporoammonic (TA) and Schaffer collateral (SC) pathways respectively. If GIRK channels are functionally overexpressed in Ts65Dn, then there is likely to be disrupted integration of distal and proximal signals as well as mistiming in hippocampal circuitry. These disruptions can explain some of the synaptic plasticity and cognitive deficits associated with Down syndrome. Synaptic $GABA_B$/GIRK currents evoked by stimulation at TA zone of Ts65Dn CA1 neurons were significantly elevated and their frequency-dependence was impaired. In contrast, GABAergic transmission at SC terminals was unaltered, indicating input specific deficits in Ts65Dn hippocampal inhibition.

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CHAPTER 2

Ts65Dn, a mouse model of Down syndrome, exhibits increased $GABA_B$ induced potassium current

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Abstract

Down syndrome (DS) is the most common nonheritable cause of mental retardation. DS is the result of the presence of an extra chromosome 21 and its phenotype may be a consequence of overexpressed genes from that chromosome. One such gene is Kcnj6/Girk2, which encodes the G-protein coupled inward rectifying potassium channel subunit 2 (GIRK2). We have recently shown that the DS mouse model, Ts65Dn, overexpresses GIRK2 throughout the brain and in particular the hippocampus. Here, we report that this overexpression leads to a significant increase (~2 fold) in $GABA_B$-mediated GIRK current in primary cultured hippocampal neurons. The dose response curves for peak and steady state GIRK current density is significantly shifted left towards lower concentrations of baclofen in Ts65Dn neurons compared to diploid controls, consistent with increased functional expression of GIRK channels. Stationary fluctuation analysis of baclofen-induced GIRK current from Ts65Dn neurons indicated no significant change in single channel conductance compared to diploid. However, significant increases in GIRK channel density was found in Ts65Dn neurons. In normalized baclofen-induced GIRK current and GIRK current kinetics no difference was found between diploid and Ts65Dn neurons, which suggest unimpaired mechanisms of interaction between GIRK channel and $GABA_B$ receptor. These results indicate that increased expression of GIRK2 containing channels have functional consequences which likely affect the balance between excitatory and inhibitory neuronal transmission.

Introduction

Mental retardation can be attributed to abnormal neural functioning caused by a spectrum of abnormalities that disrupt central nervous system (CNS) function. The cognitive deficits associated with Down syndrome (DS) are the result of expression of genes from the third copy of chromosome (Chr.) 21 and their effects on CNS function. The major neurological DS phenotypes include mental retardation, muscle hypotonia and appearance of Alzheimer disease neuropathology after age 35 (Antonarakis et al. 2004). Among the genes on Chr. 21 (Hattori et al. 2000) that might contribute to mental retardation in DS are genes located either within or in proximity to the DS critical region (DSCR) (Galdzicki et al. 2001; Toyoda et al. 2002). However, genes outside the DSCR also can be involved in the DS phenotype (Korenberg et al. 1994) and recently the concept and existence of the DSCR has been challenged (Olson et al. 2004). Although much investigation has focused on anatomic, chemical and metabolic deficiencies in DS and DS mouse models, neurophysiological abnormalities in single neurons derived from segmental trisomy mouse models (Ts65Dn, Ts16Cje and Ts1Rhr) has not been widely investigated. In this study we investigated electrophysiological properties of cultured Ts65Dn neurons to evaluate the impact that an extra gene copy of a potassium channel subunit (Kcnj6/Girk2) located within common segment of extra Chrs. in Ts65Dn, Ts1Cje, and Ts1Rhr mice has upon intrinsic neuronal properties.

The distal segment of mouse Chr. 16 is homologous to nearly the entire long arm of human Chr. 21, thus trisomic mouse models have been generated which genetically model the human condition. Full trisomy 16 and segmental trisomy 16 (Ts65Dn, Ts1Cje and Ts1Rhr) mice have been developed. Ts65Dn, and Ts1Cje mice mimic many of the

behavioral, learning and developmental deficits characteristics in DS individuals (Galdzicki et al. 2001; Galdzicki and Siarey 2003; Holtzman et al. 1996; Reeves et al. 1995; Sago et al. 1998). Protocols that induce long-term potentiation and depression (LTP and LTD) show abnormal synaptic plasticity in these mice. LTP is decreased while LTD is elevated in Ts65Dn and Ts1Cje hippocampi (Costa and Grybko 2005; Kleschevnikov et al. 2004; Siarey et al. 1999; Siarey et al. 1997; Siarey et al. 2005). In Girk2 knockout mice LTP and LTD are also abnormal (Adeniji-Adele 2004) but in the opposite direction, suggesting that the level of GIRK2 expression correlates with the level of potentiation or depression of hippocampal synapses.

Kcnj6 which encodes GIRK subunit 2 of the G-protein coupled inward rectifying potassium channel (GIRK) is located within the DSCR on human Chr. 21 (Hattori et al. 2000) and likewise within triplicated segments of mouse Chr. 16 from DS mouse models. Mammalian GIRK channels are homo- and hetero-tetramers formed from GIRK1-GIRK4 subunits and are activated by neuromodulators acting on G-protein coupled receptors (GPCR). Within the CNS, GIRK1-GIRK3 subunits contribute to the formation of functional GIRK channels with GIRK3 playing a minor role in neuronal membrane potassium conductance (Koyrakh et al. 2005). In the hippocampus GIRK1 and GIRK2 subunits are primarily localized to postsynaptic compartments and particularly found in perisynaptic and extrasynaptic regions of dendritic spines of CA1 pyramidal neurons (Drake et al. 1997; Koyrakh et al. 2005). Furthermore, tonic GIRK open channel probability increased with dendritic distance from the soma (Chen and Johnston 2005). The postsynaptic location and function of GIRK containing channels signify that these channels play an important role in synaptic function and modulation and that the

expression levels would impact intrinsic dendritic properties and the ability of neurons to properly integrate, modulate, and encode excitatory and inhibitory input. Through their contribution to the potassium conductance they can influence resting membrane potentials (Koyrakh et al. 2005; Luscher et al. 1997) and can impede neuronal excitability via shunting and slowing frequency of spike trains (Ehrengruber et al. 1997; Hille 2001).

High hippocampal GIRK expression indicates the importance that GIRK channels may have in modulating learning and memory. Indeed, reports have established that rodents lacking GIRK1 & GIRK4 subunits demonstrate defective learning and memory (Kourrich et al. 2003; Wickman et al. 2000). Our previous study (Harashima et al. 2006) showed that the presence of an extra GIRK2 gene copy in Ts65Dn hippocampus leads to elevation in GIRK2 mRNA. As a consequence GIRK2 subunit protein was overexpressed in Ts65Dn neurons. Interestingly, GIRK1 subunit protein was also overexpressed, whereas GIRK1 mRNA was normal (Girk1 gene is not localized to the Ts65Dn chromosome). These data strongly suggest that GIRK current should be greater in Ts65Dn hippocampus in comparison to diploid neurons because heterotetramers of GIRK1 and GIRK2 are the most predominant form of GIRK channel in CNS neurons. The physiological impact of GIRK1-2 channel subunit overexpression in DS has not been demonstrated. In this study we sought to reveal that GIRK2 (and consequential GIRK1) subunit overexpression leads to an increase in GIRK current which would likely impact tonic inhibitory tone. We demonstrate herein that the reported overexpression levels of GIRK channel subunits in Ts65Dn mice correlates with increases in GIRK currents and changes in intrinsic neuronal properties from hippocampal neurons cultured from the Ts65Dn neonatal mouse.

Methods

Cell Culture: Mouse hippocampal neurons were cultured in a similar manner to that described previously (Galdzicki et al. 1998). Litters from Ts65Dn mothers and diploid fathers were taken at postnatal days one and two (P1-2). Pups were decapitated, and hippocampi dissected out, after which free hippocampi were cut into pieces and incubated in 0.05% trypsin (w/v) for 10 minutes at 37ºC. Tissue was washed twice with plating media after which hippocampal cells were mechanically separated by trituration through a polished glass Pasteur pipette. Hippocampal cells were then plated on 35 mm Nunc brand dishes previously coated with 25 μg/mL poly-D-lysine and maintained at 37ºC and 5% CO_2 in the presence of plating media. One day after plating, the media was replaced with maintenance media. Plating media was composed of Neurobasal-A media with B27 supplement (Invitrogen/Gibco BRL, Carlsbad, California), fetal bovine serum (FBS, 10%), horse serum (HS, 5%) and glutamine (1%) (Biosource, Camarillo California), while maintenance media was of the same composition but without FBS or HS. Throughout the dissection and plating procedure tissue from each pup was processed separately and ploidy was determined afterwards. Mice were karyotyped with blood as described previously (Harashima et al. 2006). All recordings and the initial analysis were done without prior knowledge of the genotype.

Electrophysiological recordings: Whole-cell patch-clamp recordings of isolated hippocampal neurons were performed 10 to 20 days after plating. This age complies with

functional $GABA_B$ receptors and GIRK channels in cultured neurons (Correa et al. 2004). Pipettes were backfilled with a solution containing in mM: K-gluconate 100, KCl 20, HEPES 10, EGTA 10, $CaCl_2$ 1, Mg-ATP 4, Na-GTP 0.3, Na-phosphocreatine 7, pH 7.35. In some instances the 0.3 mM Na-GTP was replaced with 0.3 mM Li_3-GTPγS, a nonhydrolyzable GTP analog. At the time of recording, maintenance media was removed and replaced with room temperature bathing solution containing in mM: NaCl 150, KCl 4, $CaCl_2$ 2, $MgCl_2$ 2, Na-HEPES 10, glucose 10, pH 7.35. TTX (1 μM) and MK-801 or D-APV (50 μM) were added to block spontaneous firing, and NMDA mediated currents respectively. To elicit change in the potassium driving force, the bathing solution NaCl was replaced by equivalent KCl (high K^+: 60 mM). The $GABA_B$ receptor agonist baclofen was applied in both basal and high potassium solutions via a 9-barrel Rapid Solution Changer (RSC-200, Biologic, Calix, France/Molecular Kinetics, Pullman, WA, USA) positioned ~200 μm from the patched neuron. Recordings were performed in voltage-clamp configuration at a holding potential of −70 mV and data acquired by way of an EPC-7 amplifier (HEKA), filtered at 5 kHz (8-pole Bessel filter, NPI, ALA Scientific Instruments, Inc., Westbury, NY, USA), and recorded on a personal computer as per Klein et al (Klein et al. 2001). A 5 mV hyperpolarizing step from a holding potential of 5 mV was applied to estimate membrane capacitance and resistance at the initiation of whole-cell access and at intervals throughout the recording. Similarly, resting membrane potential was measured at the beginning of each recording and throughout the experiment to assess and monitor cell viability.

Data Analysis and Statistics

High potassium (60 mM) currents were determined as steady state values subtracted from basal currents measured under 4 mM extracellular potassium. When delivered under high potassium, baclofen-evoked peak and steady state currents were determined by subtraction from the value of the high extracellular potassium steady state currents. Under basal potassium, baclofen currents were subtracted from the steady state basal current. Dose-response relationships are the best least-squares fit to eq. $1/(1+ EC_{50}/[\text{baclofen}])$, and EC_{50} is concentration of baclofen, which produces 50% of the maximum possible response.

To assess the conductance of individual channels from mice of each genotype, stationary noise analysis of current recordings were performed similar to previous reports (Sciancalepore et al. 1990; Takigawa and Alzheimer 1999; Traynelis and Jaramillo 1998). Current variance from 200 ms stretches of GIRK current were plotted as a function of the mean GIRK current value for that given stretch. Data points from individual neurons were fit to the equation: $\sigma^2 = iI - I^2 / N$ where σ^2 is the current variance, i is the unitary current amplitude, I is the whole-cell current amplitude and N is the number of available channels. The value of i determined from the above equation was then related to single channel conductance by the formula: $\gamma = i/(V - E_K)$ where γ is the single channel conductance, V is the holding potential (-83 mV after liquid junction potential correction) and E_K is the Nernst potential for potassium at high potassium recording conditions (-18 mV). Channel density was then determined for each neuron by taking the ratio of estimated N and membrane capacitance. Values of i, γ, N and channel density from each neuron were averaged and analyzed for statistical significance based on ploidy.

In order to evaluate impact of ploidy on kinetics of baclofen-induced current, tau of current activation (τ_a) and deactivation (τ_d) were calculated by fitting to a single exponential function. GIRK current desensitization was determined by subtraction of peak from steady state current values (see arrow and dashed line in Figure 1b). Changes in current from peak to steady state are likely due to desensitization rather than cell dialysis. This is because only cells that showed a return to baseline upon washout were used in the analysis and long-term agonist application showed rates of desensitization comparable to short term application.

EC_{50} values were derived using the nonlinear regression—curve fit (sigmoidal dose response with variable slope) function of GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA.USA). Genotype and dose dependence was assessed using two-way repeated measures ANOVA (GraphPad Prism version 4.03). In all other cases unpaired t-tests were employed. Significance has been assigned at $p \leq 0.05$. Data are mean ± SEM unless otherwise indicated.

Results

Preliminary experiments with agonists, such as baclofen, serotonin, acetylcholine and 2-chloroadenosine, to receptors coupled to GIRK channels regularly elicited inward rectifying potassium channel in diploid and Ts65Dn neurons. However, the $GABA_B$ receptor agonist baclofen evoked the largest and most consistent current in both diploid and Ts65Dn neurons. Therefore we proceeded to focus on currents elicited by baclofen in our experiments. $GABA_B$ specificity was determined through use of CGP-55845 (2 μM) which completely blocked baclofen induced current (n=3). Voltage ramps indicate that

the reversal potential of this current approximates the Nernst potential for potassium ions as well as showing inward rectification consistent with Kir channels (Figure 1a). All passive membrane properties were similar between genotypes (see Table 1). Resting membrane potential under current clamp was not significantly hyperpolarized in Ts65Dn neurons ($p=0.08$). These results are similar to those obtained in full trisomy 16 cultured hippocampal neurons (Galdzicki et al. 1993).

Evaluation of steady state current density evoked by application of high extracellular potassium (60 mM) found no significant differences between diploid and Ts65Dn neurons (diploid -23.6 ± 3.0 pA/pF, n=41; Ts65Dn -22.3 ± 5.4 pA/pF, n=19; $p=0.81$). Likewise, membrane resistance changes upon application of high potassium decreased at similar values between genotypes (diploid 51 ± 7%, n=6; Ts65Dn 49 ± 7%, n=8; $p=0.86$). These data indicate that potassium induced inward currents do not differentially effect membrane properties of Ts65Dn neurons. Similar results were previously reported for cultured murine "full" trisomy 16 hippocampal neurons and human DS dorsal root ganglion neurons (Galdzicki et al. 1993; Nieminen et al. 1988).

Baclofen was applied under both basal and high potassium solutions. Under basal potassium concentrations and at the holding potential (-70 mV, junction potential adjusted to -83 mV) the potassium driving force is outward. We measured this outward current after application of 25 μM baclofen and found current density responses was significantly increased in Ts65Dn (1.3 ± 0.3 pA/pF, n=16) compared to diploid (0.6 ± 0.2 pA/pF, n=11) neurons ($p<0.05$). Under high potassium where the potassium driving force is inward, baclofen-induced GIRK current density of Ts65Dn neurons were significantly shifted left toward smaller concentrations of baclofen. Peak current density of baclofen-

induced GIRK current shows dose dependence that is significantly different between diploid (n=11-23) and Ts65Dn (n=9-13) neurons (two-way RM ANOVA, p=0.0002). Peak EC_{50} shifts from 4.2 μM in diploid to 0.9 μM in Ts65Dn (Figure 2a). The steady state current density was also significantly different (two-way RM ANOVA, p<0.0001). The EC_{50} shifted left from 3.0 μM in diploid to 1.0 μM in Ts65Dn (Figure 2b). The increase in current density efficacy is consistent with an increase in channel number.

Comparisons of normalized currents were also performed to determine whether $GABA_B$ receptor affinity for baclofen and/or $GABA_B$ receptor coupling with the GIRK channel was altered in Ts65Dn neurons. When the responses were normalized to the greatest current within each neuron, diploid and Ts65Dn dose responses were similar to each other (two-way RM ANOVA, p=0.08). Normalized peak EC_{50} was 2.5 μM for diploid (n=14-26) and 1.4 μM for Ts65Dn (n=9-15; Figure 2c). Normalized steady state currents were also similar across genotype (two-way RM ANOVA, p=0.19). Normalized steady state EC_{50} was 2.1 μM for diploid and 1.4 μM for Ts65Dn (Figure 2d). These EC_{50} values are comparable to those previously reported for baclofen of 2.7-3.0 μM (Sodickson and Bean 1996; 1998). Since normalized GIRK currents are similar between genotypes it is expected that coupling mechanisms between the $GABA_B$ receptor and the GIRK channel are not abnormal in Ts65Dn neurons. Likewise, the affinity for baclofen by the $GABA_B$ receptor appears to be unaltered in Ts65Dn neurons.

GIRK channel blockers were used to block the baclofen induced currents. Tertiapin-Q (50 nM), a specific GIRK channel blocker inhibited baclofen (25 μM) induced current by 46 ± 8% in diploid (n=7) and 56 ± 5% in Ts65Dn (n=12) neurons (Figure 3a). No significant difference in the percentage of block by tertiapin-Q were

found between genotypes (p=0.25). Tertiapin-Q produced a biphasic response as shown in Figure 3a. This unique response may be indicative of the use dependency of tertiapin-Q block on GIRK channels (Huang et al. 2005; Kanjhan et al. 2005). Application of Ba^{2+} (200 μM), which blocks inward rectifying potassium channels, also inhibited baclofen (25 μM) induced currents in both Ts65Dn and diploid neurons. Interestingly, the inhibitory effect of Ba^{2+} on diploid neurons was dichotic. In one subset of neurons (7 of 11) the Ba^{2+} blocked 60 ± 14% of baclofen induced current. In the other subset of diploid neurons (4 of 11), Ba^{2+} blocked 540 ± 23% of the baclofen induced current (i.e. 100% of baclofen current and 55 ± 5% of the high potassium current (Figure 3b). This dichotic effect was not seen in Ts65Dn neurons. Ba^{2+} block in Ts65Dn neurons was similar to the first subset of diploid neurons, in that block was 56 ± 9% (n=11) of baclofen current. This suggests that the overexpression of GIRK leads to a decrease in endogenously active Ba^{2+} sensitive potassium channels in a subset of hippocampal cultured neurons.

The increased GIRK current of Ts65Dn neurons may be explained increased $GABA_B$ receptor number, receptor affinity for baclofen, coupling efficiency between receptor, G-proteins, and channel and/or GIRK channel expression. We used a nonhydolyzable form of GTP, GTPγS which can directly activate GIRK channels in order to bypass the receptor and G-proteins and thus possibly discriminate between mechanisms for increased GIRK current. We replaced the GTP of the intracellular pipette solution with the equivalent amount of GTPγS. Current density responses to high potassium with GTPγS in the pipette were compared and no significant difference was found between genotypes (diploid -28.7 ± 7.6 pA/pF, n=5; Ts65Dn -33.7 ± 4.4 pA/pF, n=5; p=0.59). The high potassium current density, although greater with GTPγS in the

pipette as opposed to GTP, was not significantly different for each genotype (diploid p=0.58; Ts65Dn p=0.31). This may be the result of variable contribution of other potassium channels modulated by GTP and G-proteins (Sanchez et al. 1998; Trapp et al. 1997).

Since the use of GTPγS was unable to substantially discriminate possible explanations for the increase in GIRK current density we used stationary fluctuation analysis. An increase in channel number or density would likely be a principle cause for the increased GIRK current density and not changes in $GABA_B$ receptor or G-protein properties. The values of the single channel conductance (γ) estimated for diploid (n=12; 19.5 pS) and Ts65Dn neurons (n=8; 25.1 pS) are within range of GIRK conductance obtained from acutely dissociated rat hippocampal neurons (Takigawa and Alzheimer 1999) but slightly smaller than that estimated from excised dendritic recordings (Chen and Johnston 2005). γ is not significantly different between diploid and Ts65Dn neurons (p=0.46; Figure 4, Table 2). However, when evaluating channel number per membrane capacitance, Ts65Dn neurons show a significant increase in channel density (80%, $p<0.05$) which can explain the significant shift in EC_{50} of GIRK currents and corroborate our previous findings that GIRK channel number is increased in Ts65Dn neurons compared to diploid (Harashima et al. 2006).

We then examined the time course of activation (τ_a) and deactivation (τ_d) for the baclofen-induced GIRK current in an attempt to address the coupling between receptor and channel. We found a significant dose-dependent decrease in τ_a (speeding of activation) in both diploid (n=4-20) and Ts65Dn (n=5-12) neurons (Figure 5a; two-way RM ANOVA, $p<0.0001$ for both diploid and Ts65Dn). A significant interaction of τ_a was

found between the genotypes (two-way RM ANOVA, $p<0.05$). Bonferroni posttests showed that no individual τ_a for a separate doses was significantly different. Deactivation kinetics show significant dose dependence for both diploid (n=3-15) and Ts65Dn (n=2-9; two-way RM ANOVA, $p<0.05$) neurons (Figure 5b). No significant interaction or effect of genotype (two-way RM ANOVA, $p=0.07$ and $p=0.42$ respectively) was found. This data supports the idea that coupling mechanisms between baclofen, $GABA_B$ receptor and the GIRK channel is similar between genotypes.

The amount to which baclofen-induced GIRK currents desensitize was evaluated in a dose dependent manner at ~15 seconds (short-term); while ~1 minute (long-term) desensitization was examined for 25 μM baclofen. Short-term desensitization of baclofen-induced GIRK current density was significantly dose-dependent for Diploid (n=12-27) and Ts65Dn neurons (n=4-15; n=4-15, two-way RM ANOVA, $p<0.0001$; Figure 5c). This is similar to what has been previously reported in rat primary hippocampal cultures (Leaney 2003). Desensitization of GIRK current density also showed significant interaction (two-way RM ANOVA, $p<0.0001$) between diploid and Ts65Dn neurons. Bonferroni posttests showed that 5 and 50 μM baclofen produced significantly different desensitization rates between genotypes ($p<0.05$ and $p<0.01$ respectively). Long-term baclofen (25 μM) exposure showed no significant difference in desensitization between genotypes (diploid 27.8 ± 4.4 %, n=7; Ts65Dn 23.6 ± 2.8%, n=14; $p=0.41$). The interpretation of these findings is not clear but may reflect complex relationships between the duration of baclofen exposure and the receptor-GIRK channel assembly.

Discussion

The data presented in this report suggest that cultured hippocampal neurons from Ts65Dn neonates have elevated responses to $GABA_B$ receptor activation as evidenced by a leftward shift in the dose response curves of GIRK current density. Variance analysis of GIRK current fluctuations suggests that the leftward shift of the dose response curve can be explained by a significant increase in channel density but not by a significant change in single channel conductance.

The GIRK currents seen in Ts65Dn neurons were consistently larger than those measured in diploid neurons for all tested doses. We see no evidence for a change in the activation and deactivation kinetics of GIRK current in Ts65Dn neurons suggesting that coupling of receptor and channel remain similar to diploid neurons. Disparate desensitization responses to baclofen between diploid and Ts65Dn neurons however, may be indicative of more complex changes in GIRK current induced by changes in channel subunit ratios.

Since hippocampal neurons from GIRK2 knockout animals show significant depolarization (Koyrakh et al. 2005; Luscher et al. 1997) we evaluated the impact of the extra Ts65Dn segment, which contains GIRK2, on resting membrane properties. Resting membrane potential from each genotype was not significantly different. In full trisomy 16 hippocampal culture we also did not find changes in membrane resting potential (Galdzicki et al. 1993). Membrane resting potentials in Ts65Dn neurons may not be affected by level of GIRK function alone since the overexpression of other genes from the Ts65Dn Chr. may contribute to neuronal resting membrane potentials.

Chen and Johnston (2005) suggested that the low GIRK conductance at the soma and low surface to volume ratio would contribute little to somatic membrane properties. Yet, at the dendrites where GIRK conductance is high and surface to volume ratio is also high, GIRK expression would have profound influence on dendritic membrane properties. Within the CA1, it is at these distal synapses where the $GABA_B$ mediated potassium currents generated by perforant pathway stimulation within the stratum lacunosum-moleculare are greater than those within the more proximal stimulation of Schaffer collaterals within the stratum radiatum (Otmakhova and Lisman 2004). This poses the possibility that perforant path input at CA1 pyramidal synapse is more profoundly influenced in the Ts65Dn mouse than Schaeffer collateral input. A change in the integrative properties of pyramidal neurons is the likely result.

Plasma membrane associated GIRK1 and GIRK2 channel subunits have been identified primarily in extrasynaptic and perisynaptic regions of the postsynaptic membrane of mouse stratum radiatum. GIRK2 but not GIRK1 signal was also found within postsynaptic specializations (Koyrakh et al. 2005). More specifically, GIRK2 immunoreactivity colabeled with $GABA_B$ receptors on dendritic spines of adult rat hippocampal pyramidal neurons. This coexpression, however, was not found in dendritic shafts where $GABA_B$ receptor and GIRK2 protein were segregated (Kulik et al. 2006). Remarkably, in rat hippocampus extrasynaptic $GABA_B$ receptors show heterogenous potassium currents and may not couple Ba^{2+}-sensitive inward rectifying potassium channels, whereas synaptic $GABA_B$ receptors are homogenous and solely elicit characteristic GIRK currents (Pham et al. 1998). What an excess of GIRK1-2 expression would do to the balance between extrasynaptic and synaptic $GABA_B$-potassium channel

coupling remains to be seen, but the extra GIRK1-2 would increase the overall inhibitory tone. (Note: Since our protocol involves bath application of baclofen both synaptic and extrasynaptic receptors were activated).

An increase in GIRK current through overexpression should have profound impact on the physiology of the neuron. It could perhaps account for the abnormalities in synaptic plasticity of Ts65Dn and Ts1Cje mice (Costa and Grybko 2005; Kleschevnikov et al. 2004; Siarey et al. 1999; Siarey et al. 1997; Siarey et al. 2005) through shifts in the dynamic range of these synapses and a disruption of the balance between excitation and inhibition. Intrinsic interneuron/GABAergic tone would impinge an elevated level of $GABA_B$ receptor mediated inhibition in mice overexpressing GIRK1-2. In native systems it is likely that lower concentrations of GABA would elicit larger GIRK currents. Thus minimal GABAergic neurotransmission should have greater shunting ability.

A heteromeric $GABA_B$ receptor consisting of subunits 1 and 2 is considered necessary for metabotropic GABA signaling. $GABA_{B1}$ is responsible for binding GABA, while $GABA_{B2}$ mediates surface trafficking and G-protein coupling (Calver et al. 2001; Galvez et al. 2001; Kaupmann et al. 1998; Margeta-Mitrovic et al. 2001; 2000; Pagano et al. 2001; Robbins et al. 2001). In hippocampal primary cultures from E18 rats, $GABA_{B1}$ isoforms and $GABA_{B2}$ receptor expression matches the time dependent increases in baclofen induced potassium currents. In contrast to diffuse $GABA_B$ staining and minimal potassium current at 3 days in vitro (DIV), dendritic and punctate distribution of staining after 14 DIV paralleled the time at which maximum current density occurred. Interestingly, as the expression of the $GABA_B$ subunits developed according to DIV, the levels of GIRK1 protein remained constant suggesting that $GABA_B$ receptor expression

was independent of GIRK (Correa et al. 2004). It is therefore unlikely that increases in baclofen-induced currents are mediated by changes in $GABA_B$ receptor expression. Likewise, our data derived from variance analysis indicating increases in channel density alone can account for the shift in dose response and argue against a change in $GABA_B$ receptor expression in these cultures.

For the most part any neurotransmitter that activates a $G_{i/o}$ coupled receptor can activate GIRK channels. Indeed, in native neurons and expression systems, acetylcholine, adenosine, endocannabinoids, dopamine, GABA, glutamate, histamine, melatonin, neuropeptide Y, norepinephrine, orexins (hypocretins), opioids, serotonin, and somatostatin, have all been shown to modulate GIRK channel activity (Bunemann et al. 2001; Hoang et al. 2003; Kobayashi et al. 1996; Kofuji et al. 1995; Kreienkamp et al. 1997; Kuzhikandathil et al. 1998; Luscher et al. 1997; McAllister et al. 1999; Nelson et al. 1996; Paredes et al. 2003; Saugstad et al. 1996; Spauschus et al. 1996; Takigawa and Alzheimer 1999; Ulens et al. 1999). Therefore it is likely that any of these systems with activity in the hippocampus and CNS which activate GIRK channels would result in abnormal DS function and may contribute to neurological and cognitive phenotypes found in DS individuals. In fact, Ts65Dn mice show an increase in GIRK2 mediated hypothermic responses to a serotonin (5-HT)1A/5-HT7 receptor agonist (Stasko et al. 2006), an effect that is most likely due to the presence of the extra Girk2 gene.

In summary, hippocampal neurons derived from Ts65Dn mice show increased sensitivity to $GABA_B$ signaling with significant shift to the left in the dose dependence relationships. The kinetics of GIRK current activation, deactivation and acute desensitization were unchanged in Ts65Dn neurons suggesting that the increased GIRK

current does not affect coupling mechanisms between the $GABA_B$ receptor, G-protein and GIRK channel. The increase in GIRK current can be attributed to a ~80% increase in channel density and not significant changes in GIRK single channel conductance.

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Figure 1

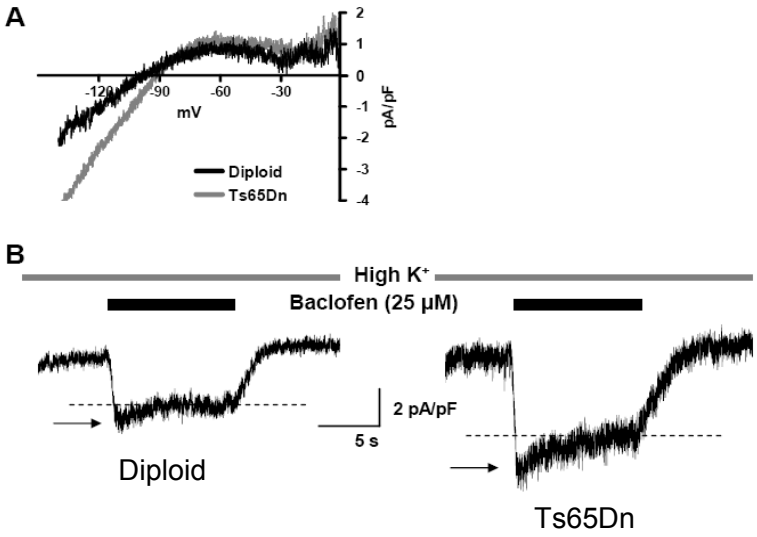

Figure 1. Baclofen induces an increase in GIRK current density in Ts65Dn in comparison to diploid neurons. (A) Current-voltage relationships of Diploid and Ts65Dn neurons show baclofen current is inwardly rectifying and the reversal potential approximates the K+ Nernst potential. IV curves were determined by subtraction of ramps with and without baclofen at basal potassium concentrations. **(B)** Example of the increased inward current density induced by 25 µM baclofen in a Ts65Dn (right) neuron compared to diploid (left).

Figure 2

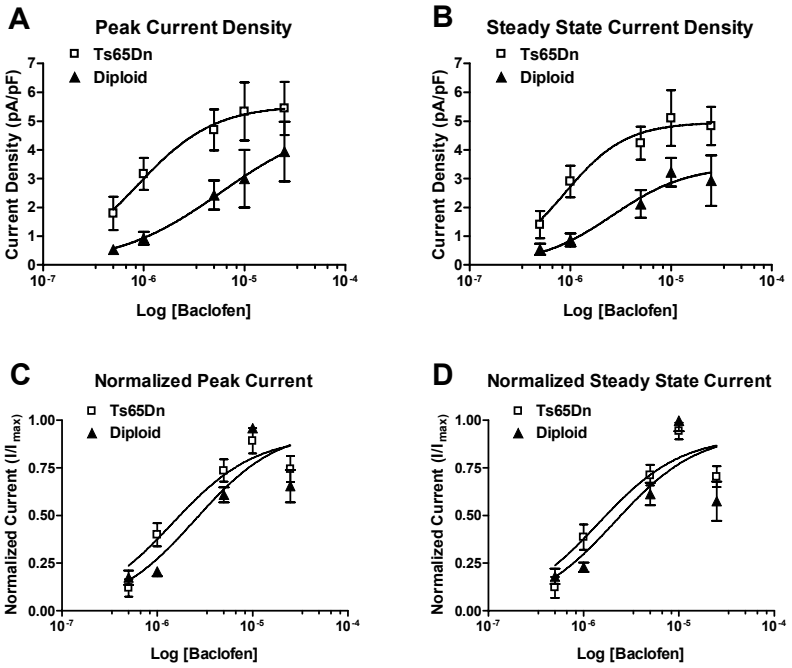

Figure 2. Baclofen-induced normalized GIRK current in diploid and Ts65Dn neurons show similar dose dependence. Peak **(A)**, denoted by arrow in (Figure 1), and **(B)** steady state, dashed line in (Figure 1), current density of baclofen-induced GIRK current show dose dependence that is significantly different between diploid and Ts65Dn neurons ($p<0.001$). Peak EC_{50} shifts from 3.2 μM in diploid to 1.4 μM in Ts65Dn and steady state EC_{50} shifts 2.1 μM in diploid to 0.9 μM in Ts65Dn. Current density is determined by dividing current by capacitance value (pA/pF). Normalized peak **(C)** EC_{50} of 2.5 μM for diploid and 1.4 μM for Ts65Dn and normalized steady state **(D)** EC_{50} of 2.1 μM for diploid and 1.4 μM for Ts65Dn are not significantly different. Peak and steady state current values were normalized to the maximum current in each cell.

Figure 3

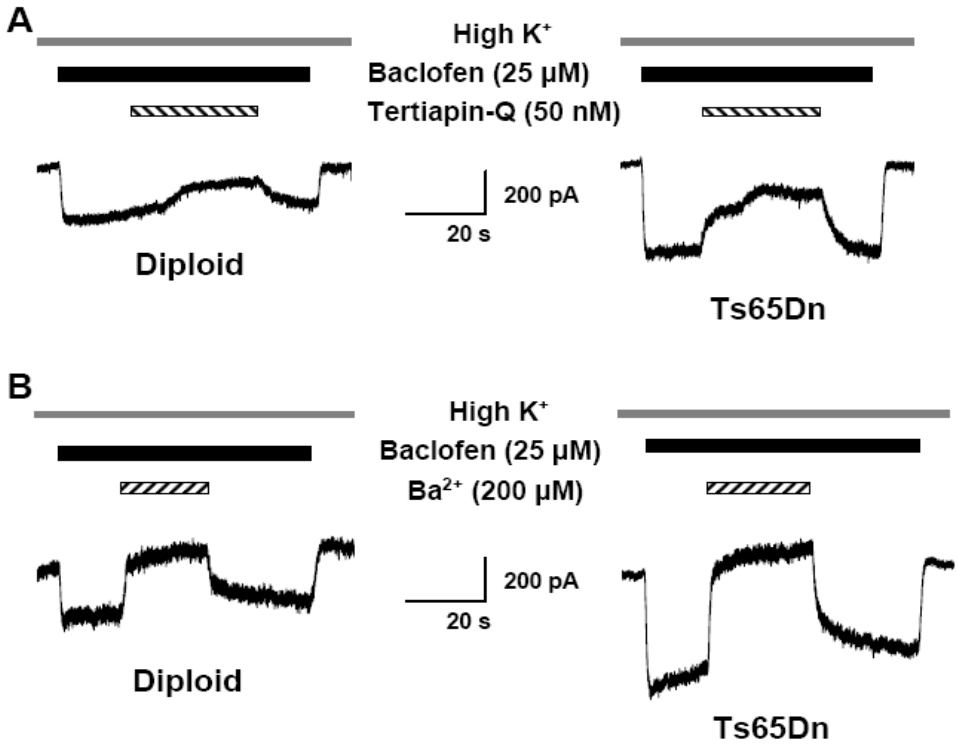

Figure 3. Block of baclofen-induced current by tertiapin-Q and Ba^{2+}. (A) Example traces showing the action of tertiapin-Q (50 nM) on baclofen-induced (25 µM) current. Tertiapin-Q blocked 46 ± 8% and 56 ± 5% of the baclofen-induced current in diploid (n=7) and Ts65Dn (n=12) neurons respectively. The biphasic block by tertiapin-Q in the Ts65Dn recording may be indicative of use dependence. **(B)** Traces demonstrating Ba^{2+} (200 µM) block of all baclofen (25 µM)-induced current. Diploid neurons responded in disparate ways to Ba^{2+}. In 4 of 11 neurons Ba^{2+} blocked all of the baclofen-induced current and part (55 ± 5%) of the high K+ current. In the other 7 of 11 neurons, the response was similar to that in Ts65Dn neurons where block was 60 ± 14% compared to 56 ± 9% in Ts65Dn neurons (n=11).

Figure 4

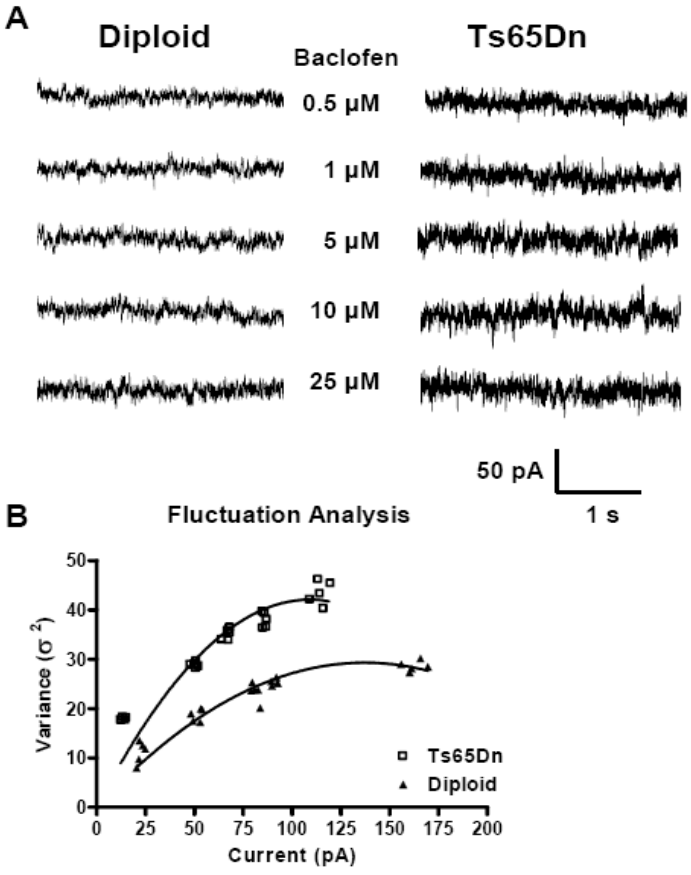

Figure 4. Single channel conductance (γ) and channel number (*N*) are similar between diploid and Ts65Dn neurons. (A) Sample recordings from increasing doses of baclofen in diploid and Ts65Dn neurons. **(B)** Current variance from 200 ms stretches of GIRK current were plotted as a function of the mean GIRK current value for that given stretch. Single channel conductance (γ) and channel number (*N*) are not significantly different between genotypes (p=0.46, p=0.74 respectively), however channel density is increased by 80% (p=0.02; see Table 2).

Figure 5

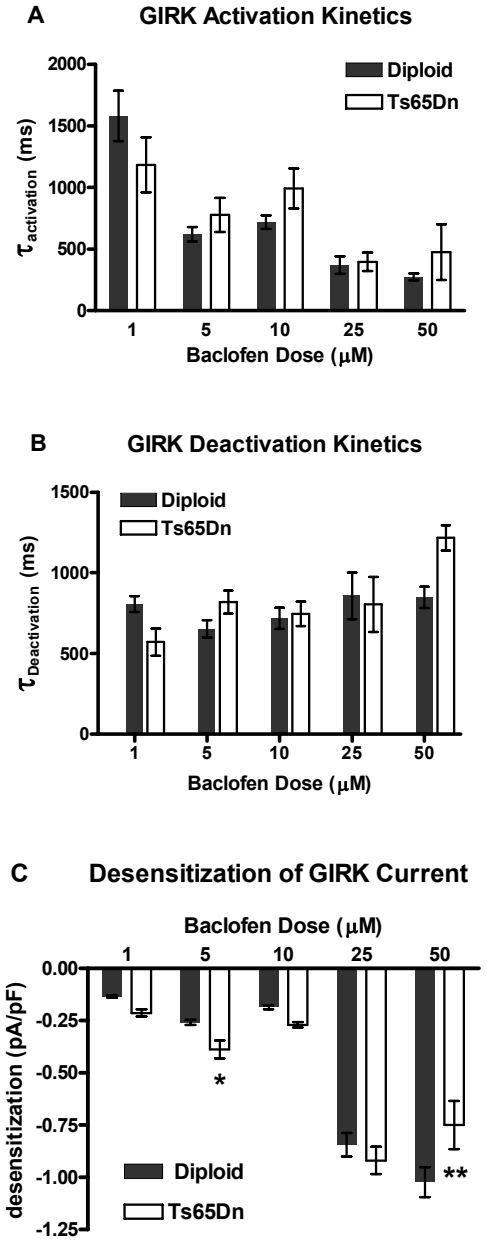

Figure 5. Normal kinetics of baclofen induced GIRK current. **(A)** The kinetics of GIRK current activation are dependent on baclofen dose ($p<0.001$), but are not significantly different between diploid and Ts65Dn neurons. **(B)** Decay kinetics show no dose dependence and are similar between ploidy. **(C)** Short-term (~15 s) desensitization of GIRK current density evaluated by the difference between peak and steady state values show dependence on baclofen dose for diploid and Ts65Dn neurons (two-way RM ANOVA, $p<0.05$). A significant interaction was found ($p<0.05$) and Bonferroni posttests indicate significant differences at 5 and 50 μM baclofen (*$p<0.05$, **$p<0.01$).

Table 1. Passive neuronal properties of diploid and Ts65Dn neurons

	Cm (pF)	Rm (MΩ)	Tau (ms)	Vm (mV)
Diploid (n=29)	26.3 ± 2.0	162.5 ± 14.8	4.4 ± 0.5	-70.7 ± 2.1
Ts65Dn (n=16)	21.8 ± 2.6	142.5 ± 20.2	3.1 ± 0.5	-75.2 ± 2.1

Membrane capacitance (Cm), membrane resistance (Rm), membrane time constant (Tau), resting membrane potential (Vm).

Table 2. Fluctuation analysis of GIRK current from diploid and Ts65Dn neurons

	γ (pS)	***N***	**Density (N/pF)**
Diploid (n=12)	19.5 ± 3.8	242.6 ± 38.9	7.1 ± 1.0
Ts65Dn (n=8)	25.1 ± 7.1	263.7 ± 50.5	12.8 ± 2.4*

Channel number (*N*), single channel conductance (γ), channel density is the ratio of channel number to membrane capacitance for each neuron analyzed. * $p=0.02$

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CHAPTER 3

Dysfunctional hippocampal inhibitory circuitry in the Ts65Dn Down syndrome mouse model: a potential role in memory consolidation

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SUMMARY

GABAergic dysfunction is implicated in hippocampal deficits of the Ts65Dn mouse model of Down syndrome. Since Ts65Dn overexpress G-protein coupled inward-rectifying potassium (GIRK) channels resulting in significant increases in $GABA_B$-GIRK function, we sought to evaluate whether genetically driven increase in GABAergic function disrupts hippocampal circuitry. Whole-cell recordings of Ts65Dn CA1 pyramidal neurons demonstrate increased spontaneous GABAergic activity and disruptions in GABAergic transmission in an input specific manner. Monosynaptic $GABA_B$-GIRK currents evoked by stimulation within the stratum lacunosum-moleculare (SLM) of Ts65Dn hippocampus are significantly elevated and their frequency-dependence is impaired. None of the properties of synaptic $GABA_B$ and $GABA_A$ mediated currents evoked by stratum radiatum stimulation appear to be affected in Ts65Dn CA1 neurons. Spontaneous inhibitory postsynaptic current (IPSC) frequency is increased ~three-fold and is not matched by an increase in miniature IPSC frequency, suggesting hyperexcitable interneurons in Ts65Dn hippocampus. An increase in $GABA_B$-GIRK at SLM inhibitory inputs can disrupt the coordination of hippocampal information processing.

INTRODUCTION

Down syndrome (DS) is the most common nonheritable cause of mental retardation with an incidence of approximately 1 in 800 live births (Nadel, 2003). DS is the result of the presence of an extra chromosome 21 and the consequences of overexpression of genes from this chromosome are considered to drive the DS phenotype (Antonarakis et al., 2004; Lejeune, 1959). One such gene found within the purported Down syndrome critical region (DSCR) is *Kcnj6* (*Girk2*), which encodes the G-protein coupled inward rectifying potassium channel subunit 2 (GIRK2). The DS mouse model, Ts65Dn, overexpresses GIRK2 throughout the brain and, in particular, the hippocampus (Harashima et al., 2006b). This overexpression leads to a significant shift in the efficacy for agonist induced GIRK current density in cultured hippocampal neurons (Best et al., 2007). *Girk2* is the only ion channel subunit encoding gene within the DSCR that is expressed at high levels within the CNS and in particular within hippocampal and cerebellar tissues where DS deficits are prominent (Galdzicki and Siarey, 2003). The extra gene copy of *Girk2* and its overexpression can plausibly explain disruptions in neuronal homeostasis and synaptic dysfunction and contribute to cognitive deficits in DS. Recent work has shown that GIRK channels may indeed be involved in some DS deficits. Chronic treatment of Ts65Dn mice with a GIRK channel antagonist (Kobayashi et al., 2003) increased neurogenesis of hippocampal neurons to levels comparable with diploid mice (Clark et al., 2006).

Within the hippocampus, GIRK channels are primarily formed as heterotetramers of alternating paired subunits of GIRK1 and GIRK2 (Bichet et al., 2003; Leaney, 2003).

Heteromeric GIRK channel complexes of GIRK1 and at least one of the other GIRK subunits are generally more functional than are homomeric channels or those composed of GIRK2 and GIRK3 (Corey and Clapham, 1998; Corey et al., 1998; Kofuji et al., 1995; Koyrakh et al., 2005; Schoots et al., 1999; Wischmeyer et al., 1997). Hippocampal GIRK channel expression pattern is graded and reflects the laminar nature of hippocampal structure. GIRK1 and GIRK2 immunoreactivity appear in strata of the dentate gyrus and CA3 and CA1 regions. Within the CA1 region, the most intense staining is found within the stratum lacunosum-moleculare (SLM) then decreases from the SLM through the distal and proximal portions of the stratum radiatum (SR) to the pyramidal cell layer (Drake et al., 1997; Liao et al., 1996). $GABA_B$ receptor subunit expression parallels GIRK channel expression where signal within the SLM is most intense in comparison to the SR and the pyramidal cell layer and stratum oriens (Lopez-Bendito et al., 2004; Sloviter et al., 1999).

This expression pattern suggests that synaptically evoked $GABA_B$-GIRK current would be larger in the SLM than that in the SR (Pham et al., 1998) and thus serve as primary inhibitory gate of temporoammonic (TA) inputs. Hippocampal CA1 distal dendrites within the SLM receive direct entorhinal cortex input via the TA pathway. This pathway is strongly activated during sensorimotor and cognitive tasks and is the principal mediator of hippocampal place field memory (Brun et al., 2002; Sybirska et al., 2000). Deficits in the TA pathway may account for the hippocampal dysfunction in Ts65Dn mice and possibly in DS individuals.

Synaptic GABA release elicits inhibitory postsynaptic currents (IPSC) mediated by $GABA_A$ and $GABA_B$ receptors and constitute fast and slow IPSCs respectively. The slow IPSC is sensitive to pertussis toxin and associated with a K^+ conductance which is attenuated by GIRK channel specific blockers and absent in GIRK2 knockout mice (Dutar and Nicoll, 1988; Huang et al., 2005; Luscher et al., 1997; Thalmann, 1987). GIRK channels are activated by agonists at Gi/o coupled receptors such as the $GABA_B$ receptor (Sodickson and Bean, 1996). This is consistent with GIRK channels as effectors for the $GABA_B$ induced slow IPSC.

DS mouse models, such as the TS65Dn mouse, display impairments in hippocampal synaptic plasticity; LTP is decreased and LTD is enhanced (Costa and Grybko, 2005; Fernandez et al., 2007; Kleschevnikov et al., 2004; Siarey et al., 1999; Siarey et al., 2006; Siarey et al., 1997). Recent studies have shown that pharmacological intervention at $GABA_A$ receptors may, in part, treat abnormal hippocampal plasticity to the point where normal physiological function is achieved under certain conditions (Costa and Grybko, 2005; Fernandez et al., 2007; Kleschevnikov et al., 2004). The results from these studies are promising in that simple blockade of inhibitory neural transmission can improve performance. However, the mechanism by which $GABA_A$ block improves function is not understood, nor is there a clear relationship between extra gene copies and $GABA_A$ergic dysfunction in DS (genes encoding $GABA_A$ receptor subunits are not on human chromosome 21, but on 4, 5, 15 and X human chromosomes (Russek, 1999)). We have previously found that Ts65Dn mice overexpress downstream effectors of $GABA_B$ signaling—GIRK channels—whose overexpression is linked to an extra *Girk2* gene copy in these mice (Harashima et al., 2006b). We also found that $GABA_B$ activated GIRK

currents are significantly elevated in Ts65Dn cultured neurons (Best et al., 2007) suggesting that mechanisms relating to GABAergic disruptions in DS mouse models may be more directly related to $GABA_B$ signaling and an imbalance in inhibitory conductances as a result of GIRK overexpression.

Given the pattern of expression of $GABA_B$ receptors and GIRK channels within the hippocampus and the fact that GIRK channels are overexpressed in Ts65Dn hippocampal neurons we hypothesize that $GABA_B$-GIRK mediated slow IPSCs would be increased. This could lead to abnormal inhibitory hippocampal function and may contribute to cognitive deficits in DS. To test this hypothesis we measured monosynaptic slow and fast CA1 IPSCs generated by stimuli within the SLM and SR. The summation of $GABA_B$ and $GABA_A$ receptor mediated currents showed diminished dynamic range in SLM but not SR of Ts65Dn hippocampus. The ratios between the charge transfer of slow and fast IPSC was significantly elevated in SLM of Ts65Dn. Furthermore, Ts65Dn CA1 pyramidal neurons showed an approximate 3-fold increase in frequency of spontaneous IPSCs with no difference in event amplitude compared to diploid.

RESULTS

Frequency dependence of $GABA_B$ mediated IPSCs

To evaluate the effect of GIRK channel overexpression in the Ts65Dn hippocampus we examined monosynaptic $GABA_B$ receptor mediated GIRK responses in CA1 pyramidal neurons to SR and SLM stimuli of Ts65Dn hippocampus. Since single stimuli did not reliably elicit observable $GABA_B$-GIRK currents (slow IPSCs), multiple

stimuli were employed. Peak amplitudes can be differentially attenuated by electrotonic distance from the soma and the absolute size of the IPSCs may not be correctly determined for stimuli delivered to distal dendrites (SLM). Because of this, and the since current integral (charge transfer or area) is less susceptible to electrotonic filtering (Spruston et al., 1993), analysis involved comparisons of charge transfer and not peak amplitudes.

To evaluate the effect that GIRK channel overexpression has on the integration of $GABA_B$ mediated signaling we used multiple stimuli at increasing frequencies and normalized the charge transfer of $GABA_B$ergic currents to responses at 5 Hz (similar to Scanziani, 2000). This analysis normalizes the response to presumably the least amount of GABA accumulation at synaptic terminals, thus providing a means to evaluate the dynamic range of GABAergic responses. Pharmacologically isolated $GABA_B$ responses to 5, 10, 20, 50 and 100 Hz in the SR and SLM were analyzed in this manner (Figure 1a-b). $GABA_B$ charge transfer at SR was comparable between diploid and Ts65Dn (Figure 2a, two-way RM ANOVA, dip n=14, Ts n=12, $p=0.10$). Within the SLM, the normalized $GABA_B$ charge transfer from diploid neurons however, was significantly increased (Figure 2b, two-way RM ANOVA, dip n=15, Ts n=11, $p<0.005$) with 100 Hz being significantly greater in diploid than Ts65Dn (Bonferroni posthoc test $p<0.05$). This suggests that there is diminished dynamic range in the summation of $GABA_B$ergic transmission in SLM of Ts65Dn. Arguably it is the accumulation of GABA released at synaptic sites which activates postsynaptic $GABA_B$ receptors and determines the response (Scanziani, 2000). Thus a limited dynamic range of responses in the SLM of

Ts65Dn hippocampi may be explained by diminished accumulation of GABA in the SLM of Ts65Dn hippocampus.

To answer whether GABA accumulation can explain this deficit we employed the GABA transporter subtype I (GAT1) specific antagonist NO-711 (10 μM) to enhance GABA accumulation. To our surprise, normalized charge transfer of $GABA_B$ responses in the presence of NO-711 were similar for both the SR and SLM for diploid and Ts65Dn neurons (Figure 2c-d, two-way RM ANOVA, SR: dip n=5, Ts n=5, p=0.36; SLM: dip n=6, Ts n=5, p=0.95). This indicates that when GABA accumulation at synaptic terminals is increased, normalized summation $GABA_B$ mediated current is similar in diploid and Ts65Dn within both the SLM and SR. This is in apparent contrast to responses without GABA uptake inhibition (Figure 2b) and indicative that there may be enhanced clearance of GABA from the extracellular space in Ts65Dn SLM. This would indicate that the limited range of responses in SLM is due to GABA uptake and not an effect of GIRK overexpression shifting the $GABA_B$ responses towards the upper range of response (nearer to plateau).

However, it is feasible that both an increase in GABA transport and a shift in the range of response due to overexpressing GIRK channels may account for the limited range of $GABA_B$ responses. An increase in functional $GABA_B$-GIRK complexes may induce compensatory changes in GABA synaptic exposure by increasing GABA transport. To test this possibility we digitally subtracted the $GABA_B$ currents with and without exposure to NO-711 (ΔNO-711). This analysis can indicate whether postsynaptic regions not normally exposed to GABA show increases in functional $GABA_B$-GIRK complex expression. Consistent with our previous SR results, normalized charge transfer

between diploid and Ts65Dn at the SR was not significantly different (Figure 2e, two-way RM ANOVA, dip n=6, Ts n=5, p=0.34). However, the normalized ΔNO-711 response to SLM stimuli was significantly greater in Ts65Dn than diploid (Figure 2f, two-way RM ANOVA, dip n=5, Ts n=5, $p<0.005$). This suggests that there is indeed overexpression of functional $GABA_B$-GIRK complexes, but that these complexes may be limited to domains not exposed to GABA under normal GABA uptake activity. Thus, the overexpression of GIRK2 in Ts65Dn shifts the distribution of $GABA_B$-GIRK functional complexes within the SLM.

Relative Contribution of Inhibition by $GABA_A$ and $GABA_B$ within the SR and SLM

Since our findings from cultured hippocampal neurons indicate that $GABA_B$-GIRK function is elevated in Ts65Dn (Best et al., 2007), we wanted to more definitively determine how GIRK channel overexpression affects GABAergic transmission. Examination of $GABA_B$ mediated currents alone may be confounded by inter-slice variability and differences in stimulus intensities from one slice to the next therefore, we normalized the values of $GABA_B$ currents to $GABA_A$ currents within the same neuron. This normalization also allows us to evaluate the relative contribution of the specific GABA receptor subtypes to overall GABA mediated transmission in diploid and Ts65Dn hippocampus.

We compared the absolute value of pharmacologically isolated $GABA_B$ charge transfer to that of the digitally isolated $GABA_A$ charge transfer from the same neurons. For stimuli delivered to the SR of diploid slices, the $GABA_B$ charge transfer was ~60% of the $GABA_A$ charge transfer while in the SLM it approached 90% (Figure 3a-inset).

Analysis of the diploid $GABA_B/GABA_A$ ratio for the range of frequencies (same as in Figure 2) showed the charge transfer from SLM stimuli to be significantly greater than that of SR (Figure 3a, two-way repeated measures (RM) ANOVA: SR n=13-14, SLM n=15, $p<0.001$). Ts65Dn showed a similar difference in the relative $GABA_B$ charge transfer between SR and SLM. Within the SR, Ts65Dn $GABA_B$ averaged ~50% while that in the SLM ~120% (Figure 3a-inset). This again was highly significant when examined for the range of stimulus frequencies (Figure 3a, two-way RM ANOVA: SR n=12, SLM n=11, $p<0.0001$), and reflects more reliance on $GABA_B$ mediated inhibition within the SLM for both diploid and Ts65Dn, possibly due to the greater distance of distal dendrites. The $GABA_B/GABA_A$ ratio within the SR was not significantly different between diploid and Ts65Dn (Figure 3a, two-way RM ANOVA, $p=0.25$) but was highly significant for SLM stimuli (Figure 3a, two-way RM ANOVA, $p<0.0005$). Thus, it is likely that the overexpression of GIRK2 and thus increased $GABA_B$-GIRK function is primarily localized to the distal dendritic arbor associated with SLM inputs and is likely to affect synapses where the integration of temporoammonic inputs occurs.

In order to further substantiate the localization of increased $GABA_B$-GIRK functional complexes, we also compared the $GABA_B/GABA_A$ ratio of $GABA_B$ responses in the presence of NO-711 (10 μM). As expected, $GABA_B$ mediated currents recorded in the presence of NO-711 (10 μM) were increased. For SR stimuli, the $GABA_B$ charge transfer with NO-711 increased to approximately 3.5 and 4.0 times the $GABA_B$ charge transfer without NO-711 and to 2.6 and 2.3 for SLM stimuli in diploid and Ts65Dn respectively. No difference was found between diploid or Ts65Dn in the $GABA_B$ charge

transfer ratios with and without NO-711 (data not shown, two-way RM ANOVA; SR dip n=5, Ts n=5, p=0.51; SLM dip n=6, Ts n=5, p=0.28).

The charge transfer ratio of NO-711 $GABA_B$ to $GABA_A$ in SR approached equivalency for both diploid (1.0 ± 0.2, n=5) and Ts65Dn (1.0 ± 0.2, n=5) while the ratio of NO-711 $GABA_B$ to $GABA_A$ in the SLM averaged 1.6 ± 0.3 for diploid (n=6) and 2.9 ± 0.5 for Ts65Dn (n=5; Figure 3b-inset). Again, the charge transfer ratios were significantly greater in the SLM compared to SR across the range of stimulus frequencies for both diploid (Figure 3b, two-way RM ANOVA, $p<0.0005$) and Ts65Dn (Figure 3b, two-way RM ANOVA, $p<0.0001$). No significant difference between SR stimuli was found between diploid and Ts65Dn (Figure 3b, two-way RM ANOVA, $p=0.66$). However, NO-711 further augments the differences between diploid and Ts65Dn charge transfer ratios within the SLM (Figure 3b, two-way RM ANOVA, $p<0.0001$). This further supports the idea that the overexpression functional $GABA_B$-GIRK complexes is localized to the distal dendritic arbor associated with SLM inputs and at extrasynaptic domains not exposed to GABA under normal GABA transporting conditions.

Normalized Charge Transfer of $GABA_A$ mediated IPSCs

An increase in the $GABA_B/GABA_A$ ratio may also be explained by reductions in $GABA_A$ responses. Therefore we wanted to examine whether $GABA_A$ transmission was affected in Ts65Dn hippocampus. Charge transfer of the digitally isolated $GABA_A$ currents acquired in Figure 3a were normalized to 5 Hz frequency (see Figure 2). The normalized responses were similar between diploid and Ts65Dn for the SR (Figure 4a, two-way RM ANOVA, dip n=13, Ts n=12, p= 0.21) while the normalized $GABA_A$

charge transfer from diploid SLM stimuli was marginally significant (Figure 4b, two-way RM ANOVA, dip n=15, Ts n=11, p=0.05) and may indicate diminished dynamic range of $GABA_A$ergic transmission in SLM of Ts65Dn similar to $GABA_B$ mediated transmission (Figure 2b).

However, digital estimates of $GABA_A$ responses may not properly approximate $GABA_A$ responses as pharmacologically isolated $GABA_A$ currents. Charge transfer of pharmacologically isolated $GABA_A$ currents (2 μM CGP55845, 20 μM CNQX, 50 μM D-APV) normalized to 5 Hz stimuli revealed no significant differences between diploid and Ts65Dn for both SR (Figure 4c, two-way RM ANOVA, dip n=12, Ts n=4, p=0.94) and SLM (Figure 4d, two-way RM ANOVA, dip n=12, Ts n=4, p=0.54). Since pharmacologically isolated $GABA_A$ frequency dependence was similar between the genotypes it is unlikely that signal integration of $GABA_A$ responses is disrupted in Ts65Dn. The marginally significant difference between Ts65Dn and diploid digitally isolated SLM responses may be a result of confounding contribution of presynaptic $GABA_B$ activity, since presynaptic $GABA_B$ receptors were not blocked when the total GABAergic responses were recorded. Variations in $GABA_A$ transmission in Ts65Dn do not have an apparent genetic basis, but may be due to compensatory mechanisms involving the balance between $GABA_A$ and $GABA_B$ mediated transmission.

Short Term Plasticity of $GABA_A$ IPSCs

To further evaluate possible differences in $GABA_A$ergic transmission we evaluated short term plasticity of only pharmacologically isolated $GABA_A$ mediated fast IPSCs (2 μM CGP55845, 20 μM CNQX, 50 μM D-APV) in CA1 pyramidal neurons by

examining peak amplitudes at varying frequencies. Five stimuli at 5, 10, 20 and 50 Hz (see Figure 1) were given within the SR and SLM of hippocampal slices from diploid and Ts65Dn mice and the ratios between the first and n^{th} peak were plotted by stimulus frequency. Previous work has shown a decrease in paired-pulse facilitation at 50 Hz of IPSCs from Ts65Dn dentate gyrus (Kleschevnikov et al., 2004). We found no such deficits in facilitation at 50 Hz in the SR (Figure 5a; two-way RM ANOVA, dip n=12, Ts n=4, p=0.49), and in fact, facilitation was significantly greater in Ts65Dn SLM than diploid at 50 Hz (Figure 5b; two-way RM ANOVA, dip n=12, Ts n=3, $p<0.0005$). This discrepancy may be due to region specific differences (dentate gyrus versus CA1 region) and possibly due to different means of evaluating short-term plasticity. Our measurement of peak amplitude was determined from baseline currents, whereas the peaks in Kleschevnikov et al. (2004) were measured from the by extrapolated decay of the previous IPSC. The most likely explanation for this discrepancy however, is that we blocked $GABA_B$ receptors while Kleschevnikov et al. did not. Presynaptic $GABA_B$ activity mediates the depression observed in short-term plasticity protocols (Otmakhova and Lisman, 2004).

$GABA_A$ short-term plasticity was unaltered in Ts65Dn SR and SLM for both 20 Hz (Figure 5c-d; two-way RM ANOVA, SR dip n=12, Ts n=4, p=0.88; SLM SR dip n=12, Ts n=4, p=0.16) and 10 Hz (Figure 5e-f; two-way RM ANOVA, SR dip n=12, Ts n=4, p=0.60; SLM SR dip n=12, Ts n=4, p=0.16). At 5 Hz, plasticity of $GABA_A$ergic IPSCs within the SR of Ts65Dn hippocampus was unaltered (Figure 5g; two-way RM ANOVA, dip n=12, Ts n=5, p=0.19). Similar to that at 50 Hz, Ts65Dn SLM showed significant differences in plasticity at 5 Hz (Figure 5h; two-way RM ANOVA, dip n=12,

Ts n=4, $p<0.0001$) with diploid showing depression (RM-ANOVA, $p<0.0001$) and Ts65Dn showing no plasticity (RM-ANOVA, $p=0.51$). These data indicate that short-term plasticity of $GABA_A$ergic stimuli within the SR is normal in Ts65Dn hippocampus at all frequencies tested. Ts65Dn SLM interestingly shows increases in short-term facilitation at 50 Hz compared to diploid but no depression at 5 Hz. This indicates a tendency for greater $GABA_A$ergic transmission in the SLM of Ts65Dn hippocampus at low and high frequencies. This stratum specific effect may be explained by differential presynaptic GABAergic function in SLM fibers that is remarkably affected by disparate frequencies.

Spontaneous Postsynaptic Inhibitory Currents

Up until now we have described disruptions of inhibitory responses to external stimuli in the Ts65Dn hippocampus but have not considered innate inhibition. Inhibitory connections are determined during the complex interplay of genetic and sensory inputs during development and circuitry maturation which may be disrupted due to abnormal synaptogenesis (Chakrabarti et al., 2007) and impaired intrinsic neuronal phenotypes. Therefore, we compared spontaneous IPSCs between diploid and Ts65Dn and found that Ts65Dn mice showed an ~300% significant increase (Figure 6a-b, $p<0.05$) in sIPSC frequency compared to diploid with no change in amplitude or decay times (see Table 1). Interestingly, the rise times of sIPSCs were significantly slower in Ts65Dn ($p<0.05$). Neurons of both diploid and Ts65Dn slices clustered into groups which exhibited event frequencies at high and low rates. Both the high and low frequency groups in Ts65Dn were significantly more frequent than the corresponding group in diploid (Figure 6c;

$p<0.0005$, $p<0.005$). The significant increase in frequency is in contrast to that seen from CA3 pyramidal neurons of Ts65Dn organotypic slice preparations which had no change in frequency (Hanson et al., 2007). An increase in frequency of spontaneous events can suggest that either, there is increased number of inhibitory synapses on CA1 pyramidal neurons or that the interneurons are more excitable, and therefore generate more frequent inhibitory events. To examine these two possibilities we depolarized the slice by using high K^+ ACSF (9 mM instead of 3 mM). Under depolarizing conditions all neurons would become more active and the frequency of events would increase in both Ts65Dn and diploid inhibitory networks.

With depolarization, sIPSC showed no significant differences in amplitude, rise and decay times (see Table 1). The frequency of events in high K^+ ACSF however, was similar between genotypes in contrast to that in normal ACSF. Additionally, the clustering of neurons into high and low frequency groups was not evident in the high K^+ ACSF. Overall, high K^+ ACSF increased sIPSC ~6 fold in diploid and only ~2 fold in Ts65Dn. Since the range of increased frequency was not equivalent between Ts65Dn and diploid it is unlikely that the increase in frequency is exclusively due to an increase in inhibitory synapses on CA1 pyramidal neurons in Ts65Dn.

To further examine if this increase in sIPSC frequency was due to hyperexcitability of interneurons or increase in inhibitory synaptic terminals, we evaluated miniature inhibitory post-synaptic currents (mIPSC) of both diploid and Ts65Dn CA1 pyramidal neurons (Figure 6c-d). We found no difference in event amplitude, rise time, and decay time between diploid and Ts65Dn (see Table 1). Furthermore, we found no significant difference in the frequency of mIPSCs between

diploid and Ts65Dn (Figure 6d; p=0.27), in contrast to the increased frequency of mIPSCs recorded from granule cells of the dentate gyrus of acute slices and the decreased frequency in CA3 pyramidal neurons from organotypic slice preparations (Hanson et al., 2007; Kleschevnikov et al., 2004). These data suggest that the increase in sIPSC frequency in Ts65Dn is not due to differences at synaptic terminals but that GABAergic interneurons display increased excitability.

Intrinsic CA1 Properties

Passive and active factors can influence the strength of synaptic inhibition in a location- and voltage-dependent manner (Hardie and Pearce, 2006). The intrinsic excitability of CA1 pyramidal neurons themselves were evaluated by examining spike threshold and burst frequencies to increasing steps of injected current (Supplemental Figure 1a). No significant differences of spike threshold, half width or maximum rise slope between Ts65Dn (n=28) and diploid (n=23) neurons were found (Supplemental Table 1). The burst frequencies likewise were similar between both genotypes (Supplemental Figure 1b; two-way RM ANOVA, p=0.98). These findings are in contrast to our previous reports from full trisomy 16 mouse cultured hippocampal neurons (Galdzicki et al., 1993).

Passive membrane properties of CA1 pyramidal neurons show an interesting *Girk2* gene dosage effect. By using Ts65Dn animals crossed with GIRK2 heterozygotes we were able to develop mice with a complete Ts65Dn background but with only two *Girk2* gene copies and thus generate mice exhibiting *Girk2* gene dosage. Resting membrane potentials show significant dependence on the number of *Girk2* gene copies

(Table 2, ANOVA $p<0.005$, post hoc trend test $p<0.005$). Estimates of cell surface area, based on membrane capacitance, are also significantly dependent on *Girk2* gene dosage (Table 2, ANOVA $p<0.0001$, post hoc trend test $p<0.0001$).

The effect of GIRK channels on resting membrane potentials has been shown previously where neurons from knockout animals were significantly depolarized compared to wildtype (Koyrakh et al., 2005; Luscher et al., 1997). Likewise it has been suggested that the subcellular localization of GIRK channels likely has a greater effect on membrane potentials where the surface to volume ratio is greater such as within the dendritic arbor as opposed to the cell soma (Chen and Johnston, 2005). Therefore, since we see a gene-dosage effect on resting potentials when recording from the soma, the effect of *Girk2* gene copy number is greater in the dendritic arbor and thus Ts65Dn dendrites are likely more hyperpolarized than what we observe at the soma, particularly because ambient GABA is likely to be increased in Ts65Dn as evidenced by the increase in spontaneous IPSC frequency (Figure 6). Passive hyperpolarization through changes in resting membrane potentials can affect tonic activity of voltage-dependent channels and Mg^{2+} block of NMDA receptors and thus dramatically influence NMDA dependent synaptic plasticity. Furthermore, active hyperpolarization through opening of GIRK channels can affect spike generation and summation of excitatory inputs thus increasing the effects that overexpression of GIRK channels may have on hippocampal signal processing (Ehrengruber et al., 1997; Takigawa and Alzheimer, 2003).

DISCUSSION

Dysfunctional hippocampal processes in the Ts65Dn DS mouse model have been improved through $GABA_A$ receptor antagonists suggesting that GABAergic transmission is defective in DS (Costa and Grybko, 2005; Fernandez et al., 2007; Kleschevnikov et al., 2004). These reports are particularly intriguing since there is no direct genetic evidence for $GABA_A$ receptor abnormalities in this DS mouse model as there is for an effector of $GABA_B$ receptor signaling—the GIRK channel (Best et al., 2007). Thus we evaluated responses to hippocampal GABAergic stimulation with a particular focus on the genetic contribution that GIRK2 containing channels have on inhibition in the Ts65Dn mouse. CA1 pyramidal neurons responded disparately to GABAergic transmission in a region specific manner. All responses to stratum radiatum (SR) stimulation were similar between diploid and Ts65Dn, whereas stratum lacunosum-molecular (SLM) stimuli evoked significantly different responses. The frequency-dependence of $GABA_B$-GIRK currents was impaired in Ts65Dn SLM. Likewise the ratio of monosynaptic $GABA_B$-GIRK currents to $GABA_A$ currents was significantly elevated in Ts65Dn SLM. The frequency dependence of monosynaptic $GABA_A$ currents appeared to be normal ion Ts65Dn SLM, yet short-term plasticity of SLM evoked $GABA_A$ currents was significantly different from diploid at 5 and 50 Hz. Moreover, the frequency differences of spontaneous and miniature IPSCs suggest that interneurons in Ts65Dn hippocampus are more excitable than in diploid. Overall, these findings indicate that GABAergic dysfunction in Ts65Dn hippocampus can be in part related to genetically driven overexpression of GIRK2 containing channels, however other developmental and genetic factors may also play a role.

Interneuron activity and slow IPSCs

Different classes of GABAergic interneurons terminate in different regions of the CA1 pyramidal cell dendrites. With bipolar stimulating electrodes placed in the SR we are primarily exciting axon terminals of horizontal and radial trilaminar cells as well as bistratified interneurons. Conversely, stimulating electrodes placed in the SLM would be associated with neurogliaform and oriens lacunosum-moleculare (O-LM) interneurons whose axon terminals are restricted to the SLM (Freund and Buzsaki, 1996; Price et al., 2005). The IPSCs we observe in this report are likely due to recruiting multiple interneuron fibers of the various interneurons subtypes and not to individual interneuron activation. Single interneurons reliably elicit $GABA_A$ mediated fast IPSCs but alone are generally not sufficient to induce slow IPSCs in hippocampal pyramidal neurons (Scanziani, 2000; however see Thomson and Destexhe, 1999). In the presence of GABA uptake inhibition, repetitive firing of an individual interneuron reliably elicits slow IPSCs (Scanziani, 2000) suggesting that GABA uptake mechanisms preclude slow IPSCs and that GABA spillover onto extra- or perisynaptic $GABA_B$ receptors are responsible for such currents.

Postsynaptic membrane associated GIRK1 and GIRK2 channel subunits have been identified primarily in extrasynaptic and perisynaptic regions of mouse stratum radiatum (SR). GIRK2 but not GIRK1 was also found within postsynaptic specializations (Koyrakh et al., 2005). This suggests that within the SR, less functional homomeric GIRK2 (or heteromeric GIRK2-3) channels exists at synaptic sites and the more functional heteromeric GIRK1-2 channels surround synapses. This distribution of GIRK

channel isoforms is consistent with the need for GABA spillover to generate slow IPSCs. Dendritic shafts also showed immunoreactivity to GIRK2 when opposed by symmetric (putative GABAergic) terminals in both the SR and SLM (Kulik et al., 2006). This shaft localization, however, was not associated with $GABA_{B1}$ suggesting that the receptor and channel are unlikely to be coupled in dendritic shafts. In contrast, GIRK2 immunoreactivity co-labeled with $GABA_{B1}$ on dendritic spines preferentially localized around asymmetric synapses (Kulik et al., 2006). This colocalization of both $GABA_{B1}$ and GIRK2 suggests functional coupling at perisynaptic regions of glutamatergic terminals. Shunting of excitatory currents may therefore be maximized in this localization by spillover from nearby GABAergic terminals.

To generate slow IPSCs a network of interneurons may need to coordinate firing so that GABA accumulation is sufficient to activate $GABA_B$ receptors. Synchronous oscillation by interneuron networks has been shown to be sufficient to effectively induce $GABA_B$ activity in cultured rat hippocampal slices (Scanziani, 2000). Interestingly oscillation frequency of interneuronal networks is dependent on $GABA_B$ function. Blocking $GABA_B$ activity increased the oscillatory frequency and enhancing $GABA_B$ activity through inhibition of GABA transport decreased the frequency (Scanziani, 2000). If CA1 pyramidal neurons influence interneuron oscillations then with the increase in $GABA_B$ response in Ts65Dn, we would expect a decrease in the interneuron oscillatory frequencies. A decrease in interneuron oscillations may also occur if there are increased $GABA_B$ergic responses in Ts65Dn interneurons themselves. At the present time, we are not aware of any direct evidence that Ts65Dn mice or DS individuals have altered oscillatory frequencies such as theta or other rhythms.

Possible disruptions in network timing

It is evident that the relative timing in pre- and postsynaptic neuron activity can determine the strength of synaptic connections (Bi and Poo, 1998; Markram et al., 1997). In particular, proper gating of temporoammonic (TA) input by the trisynaptic circuit is dependent on the timing of theta oscillations. In order for TA generated EPSPs to reach CA1 pyramidal somas, Schaffer collateral input must be phase shifted by one-half theta frequency (Ang et al., 2005). Furthermore, the strength and timing of Schaffer collateral inputs determines whether TA initiated CA1 dendritic spikes propagate beyond the SLM and initiate firing (Jarsky et al., 2005). By comparison, stimulation of the TA pathway has been shown to block CA1 pyramidal neuron spikes driven by Schaffer Collateral (SC) stimuli (Dvorak-Carbone and Schuman, 1999). This spike blocking property of the TA pathway is dependent on $GABA_B$ stimulation and is most efficacious when timed with the peak of hyperpolarizing current (Dvorak-Carbone and Schuman, 1999) suggestive of a role for GIRK channels in shunting excitatory input. Interestingly, the relative metaplastic state of synapses within the SLM demonstrated variable efficacy in blocking SC driven spikes. When the TA pathway was potentiated by an LTP inducing protocol, the ability to block SC driven spikes was enhanced, likewise, LTD protocols within the SLM decreased the efficacy to block spikes (Remondes and Schuman, 2002). Furthermore, stimulation of TA fibers was also able to impede the potentiation of SC-CA1 synapses by LTP protocols (Remondes and Schuman, 2002).

The changes we observe in GABAergic transmission can disrupt the timing of direct TA entorhinal projections onto CA1 pyramidal neurons as well as indirect

entorhinal projections via the hippocampal trisynaptic circuit. Since TA input to CA1 maintains place fields (Brun et al., 2002) the deficits in spatial memory by Ts65Dn mice (Demas et al., 1998) may be due to changes in inhibitory regulation of CA1 pyramidal neurons. Furthermore, because interneuron activity strongly regulates spike timing and rhythmic activity (Bacci and Huguenard, 2006; Wang and Buzsaki, 1996), any change in response to GABAergic signaling would disrupt the control of timing and rhythmicity by interneurons. Mistimed activity in Ts65Dn hippocampus can explain the abnormal synaptic plasticity (Siarey et al., 1999; Siarey et al., 1997) and blockade of $GABA_A$ receptors could restore timing such that plasticity resembles diploid (Costa and Grybko, 2005; Fernandez et al., 2007; Kleschevnikov et al., 2004).

The synaptic strength of Ts65Dn hippocampus may be further affected by changes in spike-timing dependent plasticity of both pyramidal cells and interneurons. Activity at $GABA_B$ receptors can block distal dendritic spikes and delay backpropagating spikes in CA1 pyramidal cells (Leung and Peloquin, 2006). Elevated $GABA_B$ function would clearly enhance the spike blocking efficacy and increase the delay of backpropagating spikes. Spike-timing dependent plasticity of GABAergic synapses has been demonstrated to diminish the function of the neuronal K^+/Cl^- cotransporter (KCC2) which subsequently alters the GABA reversal potential (E_{GABA}) to be more depolarizing (Woodin et al., 2003). Depolarized E_{GABA} is particularly relevant to timing issues given that interneuron synchronization is dependent on spike afterhyperpolarization (AHP) amplitude being above the $GABA_A$ synaptic reversal potential (Wang and Buzsaki, 1996).

An intriguing interplay between AHP and $GABA_B$-GIRK current may be at work in region specific domains of Ts65Dn CA1 that is likely to affect plasticity of these synapses. The magnitude and mechanism of AHP within CA1 pyramidal neurons is dependent on synapse location. Stimuli given within SR results in relatively small AHP that is primarily mediated through ZD7288 sensitive I_h channels, whereas, stimuli within the SLM induce relatively large AHP that is partially sensitive to ZD7288 but mostly mediated by $GABA_B$-GIRK (Otmakhova and Lisman, 2004). This AHP magnitude difference between SR and SLM, parallels data in this current report that $GABA_B$-GIRK mediated slow IPSCs are greater in the SLM. Furthermore, NMDA currents in the SLM are effectively attenuated by postsynaptic $GABA_B$ activity (Otmakhova and Lisman, 2004). This substantiates previous results indicating that $GABA_B$-mediated slow IPSCs are sufficient to inhibit NMDA receptor-mediated excitatory postsynaptic currents (EPSC) in dentate molecular layer interneurons (Mott et al., 1999). Interestingly, $GABA_B$ mediated GIRK channel activation also suppresses SR AHP through transient deactivation of AHP-induced cation current (I_h) (Takigawa and Alzheimer, 2003). GIRK channels also attenuate the amplitude and summation of EPSPs within the SR (Takigawa and Alzheimer, 2002; Takigawa and Alzheimer, 2003).

Taken together, the present results show previously uncharacterized differential impact of $GABA_B$ and $GABA_A$ stimuli in hippocampal circuitry. Moreover, an increased $GABA_B$-GIRK response in CA1 pyramidal neurons is likely to disrupt critical factors contributing to abnormal coordinated firing and plasticity. Given the important role of GIRK2 in the hippocampus, GIRK2 represents a novel therapeutic target for the treatment of some cognitive dysfunction associated with Down syndrome.

EXPERIMENTAL PROCEDURES

Animals

Ts65Dn and control diploid littermates were bred to have the mixed genetic background C57BL/6JEi×C3H/HeSnJ as used in our previous studies (Harashima et al., 2006b; Siarey et al., 1997). *Girk2* null mice were generated as previously reported (Signorini et al., 1997). The disomic-Ts65Dn/*Girk2*$^{(+/+/-)}$ mouse that is diploid for the *Girk2* gene and trisomy for the rest of the Ts65Dn segment of chromosome 16 was generated as follows. Males heterozygous for the *Girk2* gene (heterozygous-Diploid/*Girk2*$^{(+/-)}$) were bred with Ts65Dn females to yield the following mice: homozygous-Diploid/*Girk2*$^{(+/+)}$; heterozygous-Diploid/*Girk2*$^{(+/-)}$; disomic-Ts65Dn/*Girk2*$^{(+/+/-)}$ and Ts65Dn/*Girk2*$^{(+/+/+)}$, such that mice had one, two, two and three copies of *Girk2* gene respectively (Harashima et al., 2006a). The mice used in this research were genotyped by fluorescence *in situ* hybridization and PCR screening using tail DNA as described before (Strovel et al., 1999; Wickman et al., 1998). Mice were maintained under a 12-h light/dark cycle and fed standard laboratory food (following the NIH guidelines). All protocols were approved by the USUHS Institutional Animal Care and Use Committee.

Slice Preparation

Mice, 2-3 weeks old, were anesthetized, decapitated and the brain was rapidly removed and placed in ice cold (~4°C) cutting artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 3, $CaCl_2$ 2, NaH_2PO_4 1.25, $MgSO_4$ 5, $NaHCO_3$ 26,

d-glucose 10, bubbled with a mixture of 95% O_2/5% CO_2. Parasagittal slices, 400-µm thick, were cut on a Lancer Vibratome (Vibratome series 1000; Vibratome, St. Louis, Missouri USA) and transferred to a warmed (~37°C) solution of 50% slicing ACSF and 50% recording ACSF (the same composition as cutting ACSF, but $MgSO_4$ at 1 instead of 5 mM) bubbled with a mixture of 95% O_2/5% CO_2. After 20 minutes they were transferred to room temperature (~21°C) O_2/CO_2 bubbled recording ACSF where they were maintained for at least 1 hour before recording.

Electrophysiology

Slices were placed in a recording chamber containing bubbled recording ACSF on the stage of an upright Zeiss FS-1 microscope (Carl Zeiss Microimaging Inc., Thornwood, NY). Using a Photonics IR camera, CA1 pyramidal neurons were identified and a whole-cell patch-clamp configuration was obtained with a borosilicate patch pipette of resistance 3-5 MΩ containing (in mM): K-gluconate 130, KCl 15, HEPES 5, EGTA 1, Mg-ATP 4, Na-GTP 0.3 with pH adjusted to ~7.3 with KOH. A 5 mV hyperpolarizing step from a holding potential of -70 mV was applied to estimate membrane capacitance and resistance at the initiation of whole-cell access and at intervals throughout the recording. Similarly, resting membrane potential (current-clamp: I=0) was measured at the beginning of each recording and throughout the experiment to assess and monitor cell viability. Recordings were performed in voltage-clamp and current-clamp configurations and data acquired by way of an Axopatch 200A or 200B amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA), filtered at 3 kHz (8-pole Bessel filter,

NPI, ALA Scientific Instruments, Inc., Westbury, NY, USA), and recorded on a personal computer using Clampex acquisition software (Axon Instruments).

After perfusion of 20 μM CNQX and 50 μM D-APV (to block AMPA/Kainate and NMDA receptor mediated currents) and prior to stimulation protocols, spontaneous inhibitory post-synaptic currents (sIPSC) were recorded from neurons held at -70 mV under a gap free protocol and later visually inspected and detected offline using MiniAnalysis 6.0 (Synaptosoft, Decatur GA.). sIPSC were eliminated by 20 μM bicuculline (specific $GABA_A$ receptor antagonist) in both diploid (n=4) and Ts65Dn (n=5) slices indicating that events were $GABA_A$ mediated. Spontaneous events were analyzed for amplitude, rise (10-90%) and decay (90-10%) times. Event frequency was determined by the number of events per time from the first to the last event detected. On average, 125 events from each neuron were used in the analysis. Miniature IPSCs (mIPSC) were also recorded at -70 mV in perfusate of 1 μM TTX (to block action potentials and thus spontaneous IPSCs), 20 μM CNQX and 50 μM D-APV and analyzed as described above for spontaneous IPSCs.

In the presence of 20 μM CNQX and 50 μM D-APV GABAergic currents were elicited by current stimulation with a concentric bipolar platinum electrode placed within the stratum radiatum (SR) to stimulate interneuron fibers associated with the Shaffer collateral (SC) pathway or by a second stimulating electrode placed within the stratum lacunosum-moleculare (SLM) opposite the patched CA1 pyramidal neuron to stimulate interneuron fibers associated with the temporoammonic (or perforant) pathway. Stimulation electrodes were placed in such a way as to avoid co-stimulation of the same

terminals; that in the SR near the border of the pyramidal cell layer and that in the SLM within ~100 μm of the hippocampal fissure. Multiple stimuli were given at an intersweep interval of 20 seconds. Three sweeps of each condition were averaged and later used for analysis.

For evaluation of the relative contribution of GABAergic currents, the ratio between pharmacologically isolated $GABA_B$ currents and digitally subtracted $GABA_A$ mediated currents were used. $GABA_B$ mediated GIRK currents were pharmacologically isolated from general GABAergic currents by application of 20 μM bicuculline to the existing 20 μM CNQX and 50 μM D-APV containing ACSF. The absolute values of $GABA_B$ charge transfer for 5 Hz stimulation was 27.7 ± 2.9 pC for SR and 25.7 ± 3.2 pC for SLM in diploid and 26.0 ± 3.2 pC for SR and 18.9 ± 2.2 pC for SLM in Ts65Dn. Digitally subtracted $GABA_A$ traces were established by subtracting the pharmacologically isolated $GABA_B$ traces from general GABAergic traces (those recorded without bicuculline perfusion). Trace subtraction and comparison were performed only in traces of identical stimulus intensity and holding potentials. Specificity for $GABA_B$ mediated currents was tested through use of 2 μM CGP55845 (a $GABA_B$ receptor specific antagonist) which completely inhibited slow IPSCs within 5 minutes perfusion (n=16). Likewise slow IPSCs were blocked by GIRK channel blockers tertiapin-Q (100 nM, n=5, 55 ± 0.1% at 15 minutes) and SCH23390 (10 μM, n=6, 54 ± 0.1% at 12 minutes) (Kuzhikandathil and Oxford, 2002). Incomplete block of slow IPSCs by GIRK channel blockers may be due to heterogenous $GABA_B$ postsynaptic currents, partial block, or use dependence (Best et al., 2007; Kanjhan et al., 2005; Pham et al., 1998; Tabata et al., 2005).

Peak amplitudes of pharmacologically isolated $GABA_A$ mediated IPSCs (20 μM CNQX, 50 μM D-APV, and 2 μM CGP55845) were normalized to the amplitude to the first peak in a series of 5 stimuli at 5, 10, 20, and 50 Hz in order to evaluate short-term plasticity of fast IPSCs.

Action potentials were elicited from CA1 pyramidal neurons at increasing steps (10 pA) while current-clamped at -70 mV. Spike threshold was evaluated by visually identifying the voltage at which maximum acceleration occurred within the rising phase of the first spike of each neuron. Spike amplitude, half-width, and maximum rise slope were determined as with Galdzicki et. al. (1993) by use of pClamp software (Axon Instruments).

Unpaired two-tailed t-tests were performed for statistical significance unless otherwise stated. Significance was set at $p<0.05$. CNQX, D-APV, Bicuculline methiodide, CGP55845, SCH23390, TTX and tertiapin-Q were all purchased from Tocris (Ellisville, Missouri USA). NO-711 was purchased from Sigma-Aldrich (St. Louis, Missouri USA).

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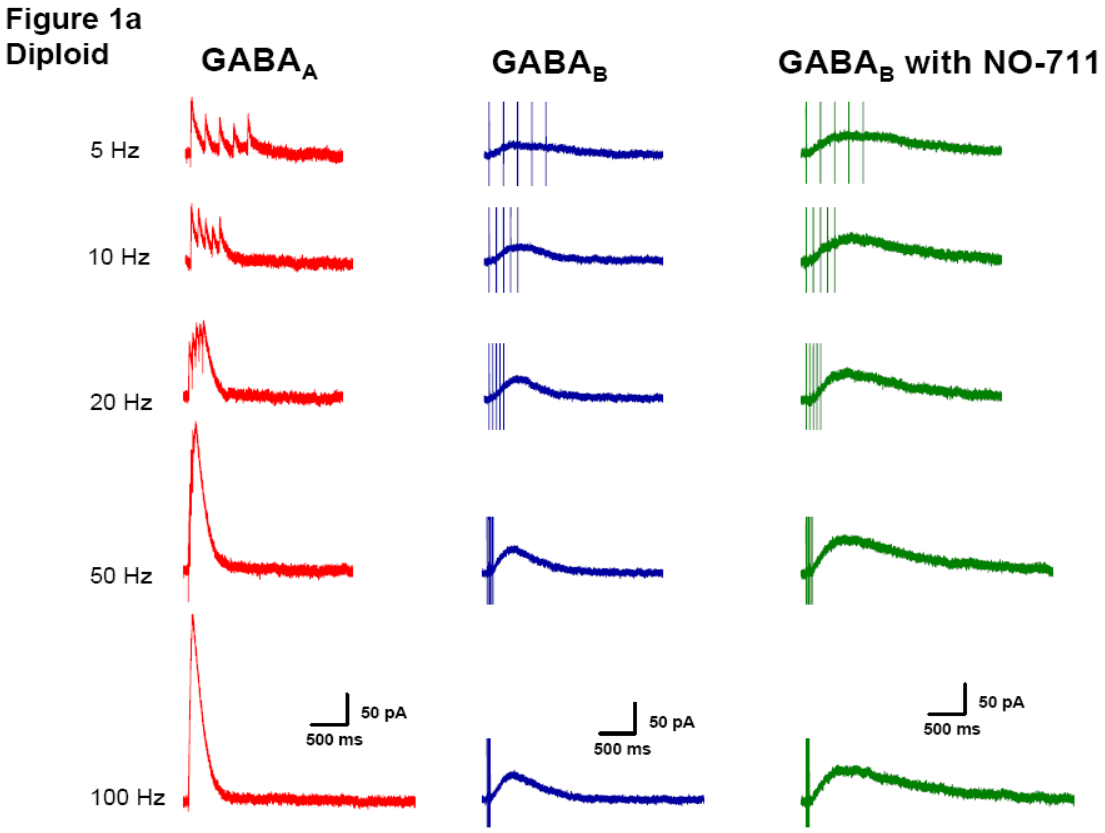


Figure 1. GABAergic responses by CA1 pyramidal neurons to stimuli at increasing frequencies.
Representative recordings from an individual diploid **(A)** and Ts65Dn **(B)** neuron in response to SLM stimulation at 5, 10, 20, 50 and 100 Hz (Vhold = -35 mV). $GABA_A$ (red), $GABA_B$ (blue) and $GABA_B$ with NO-711 (green) recordings were acquired follows: After recording general GABAergic currents in the presence of 20 μM CNQX and 50 μM D-APV, bicuculline (20 μM) was then perfused to isolate pure $GABA_B$ mediated GIRK currents. After which, NO-711 (10 μM), a GAT1 GABA transporter antagonist, was then perfused. $GABA_A$ currents were isolated by digital subtraction of current traces in the absence or presence of $GABA_A$ receptor inhibitor. Note the relative increase of GABAergic currents which tends to plateau at 50 Hz and the effect of GABA uptake inhibition (NO-711) on $GABA_B$ currents. Ts65Dn recordings have been scaled to the peak amplitude of the first $GABA_A$ IPSC so as to better compare $GABA_B/GABA_A$ ratios. Traces are the average of 3 sweeps under each condition. Stimulus intensity was identical throughout recording and stimulus conditions for each neuron respectively. Stimulus artifacts have been truncated for clarity.

Figure 1b
Ts65Dn

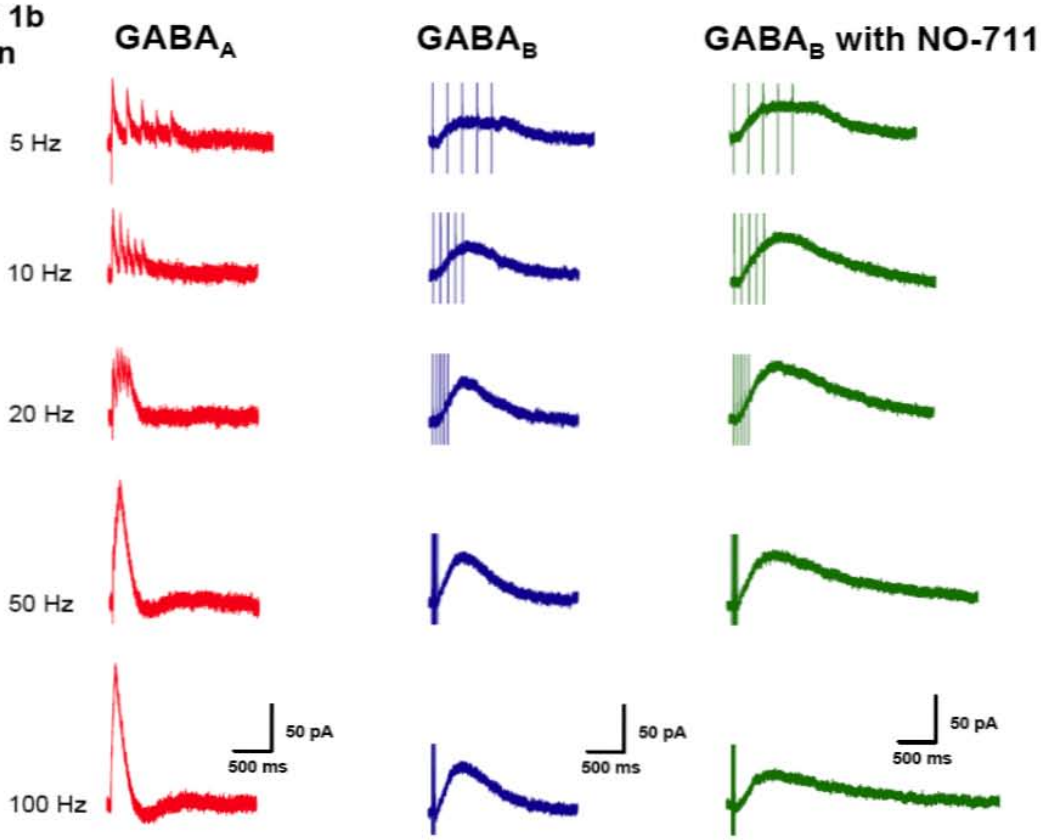


Figure 3

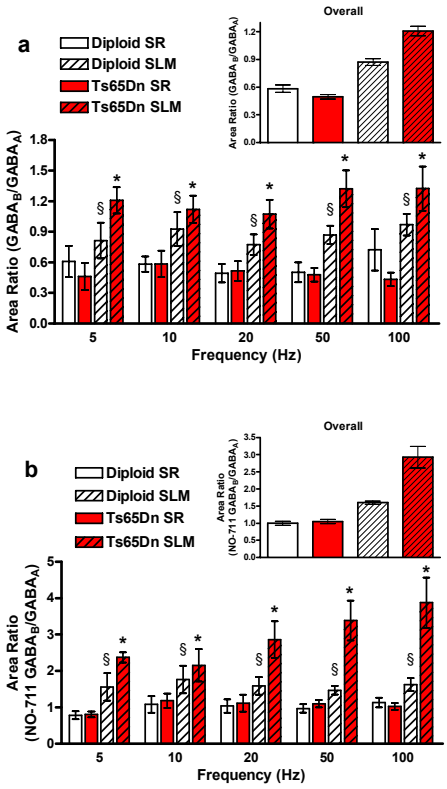


Figure 3. GABA$_B$/GABA$_A$ charge transfer ratios show input specific dependence.

a. The ratio between charge transfer of GABA$_B$ and GABA$_A$ receptor mediated currents (the ratio of total area under the GABA$_B$ or GABA$_A$ current curve for the entire train of five synaptic stimuli) is significantly greater for SLM (hatched bars) than SR (solid bars) stimuli in both diploid (white bars; two-way RM ANOVA: SR n=13-14, SLM n=15, $p<0.001$) and Ts65Dn neurons (red bars; two-way RM ANOVA: SR n=12, SLM n=11, $p<0.0001$). There is no difference between diploid and Ts65Dn for SR stimuli, however, Ts65Dn SLM shows significantly higher charge ratios than diploid SLM (two-way RM ANOVA, $p<0.0005$). The inset shows the overall average when combining all frequencies.

b. Inhibition of GABA uptake by NO-711 (10 μM), although increasing the magnitude of GABA$_B$/GABA$_A$ ratio, does not change the overall pattern of GABAergic contribution by GABA$_B$ or GABA$_A$ currents. SLM inputs exhibit greater NO-711 GABA$_B$/GABA$_A$ ratios than SR for both diploid and Ts65Dn (two-way RM ANOVA; dip, SR n=5, SLM n=6, $p<0.0005$; Ts SR n=5, SLM n=5, $p<0.0001$) with no difference between diploid and Ts65Dn at SR inputs. However, it appears that NO-711 enhances the difference between diploid and Ts65Dn at SLM inputs (two-way RM ANOVA, $p<0.0001$). Frequency dependence of Ts65Dn SLM ratios was not significant (ANOVA, $p=0.13$). The inset shows the overall average when combining all frequencies.

Figure 4

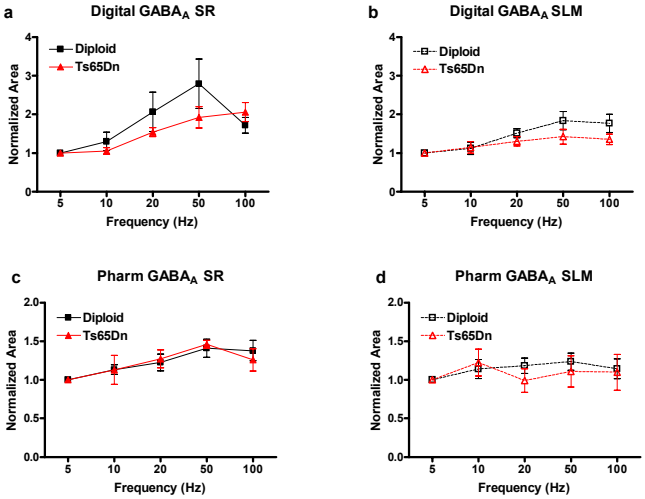


Figure 4. Frequency dependent integration of in digitally and pharmacologically isolated GABA$_A$ currents in CA1 pyramidal neurons.
a. Charge transfer normalized to 5 Hz stimulation in the SR show no differences between diploid (black) and Ts65Dn (red) digitally isolated GABA$_A$ currents.
b. SLM stimuli (open symbols, dashed lines) indicate marginally significant less signal integration for digitally isolated GABA$_A$ currents of Ts65Dn compared to diploid (two-way RM ANOVA, dip n=13, Ts n=12; p=0.05).
c,d. Pharmacologically isolated (2 μM CGP55845, 20 μM CNQX, 50 μM D-APV) GABA$_A$ charge transfer in response to SR or SLM stimuli is similar between diploid and Ts65Dn.

Figure 5

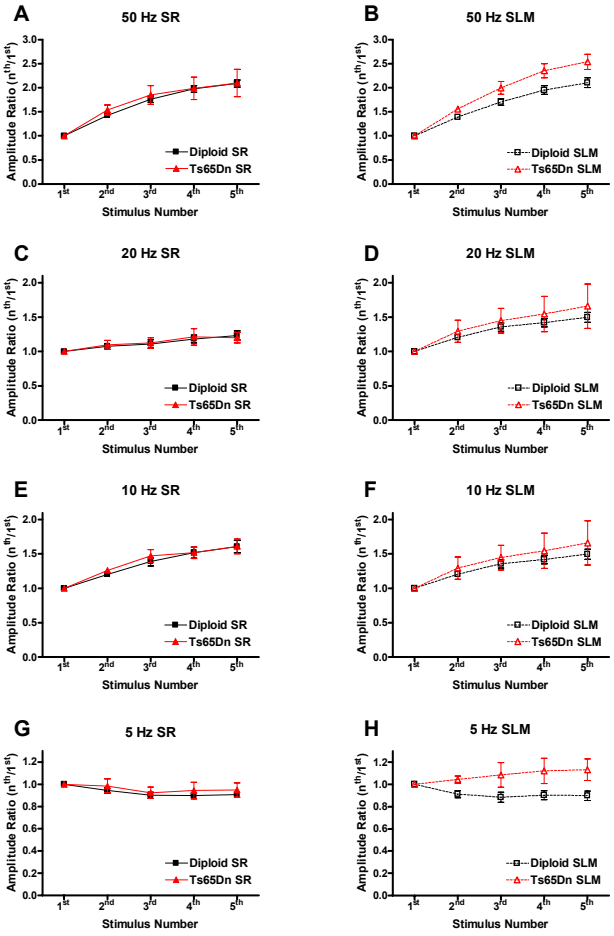


Figure 5. Short-term facilitation of $GABA_A$ IPSCs is normal at SR but not SLM inputs in Ts65Dn CA1 pyramidal neurons.

Pharmacologically isolated $GABA_A$ peak amplitudes were normalized to the 1st peak in a train of five stimuli at increasing frequencies and examined for short-term plasticity.

A. No differences in facilitation at 50 Hz stimulation were found between diploid (black squares) and Ts65Dn (red triangles) neurons at SR inputs.

B. Facilitation at 50 Hz stimulation in the SLM of Ts65Dn is significantly significantly stronger than in diploid SLM (two-way RM ANOVA, dip n=12, Ts n=3, $p<0.0005$).

C-F. At 20 and 10 Hz, facilitation was similar between diploid and Ts65Dn at both SR and SLM inputs

G. Short-term plasticity at 5Hz stimulation within the SR was again similar between genotypes.

H. SLM stimuli at 5 Hz elicited short-term depression in diploid but not in Ts65Dn neurons (two-way RM ANOVA, dip n=12, Ts n=4, $p<0.0001$).

Figure 6

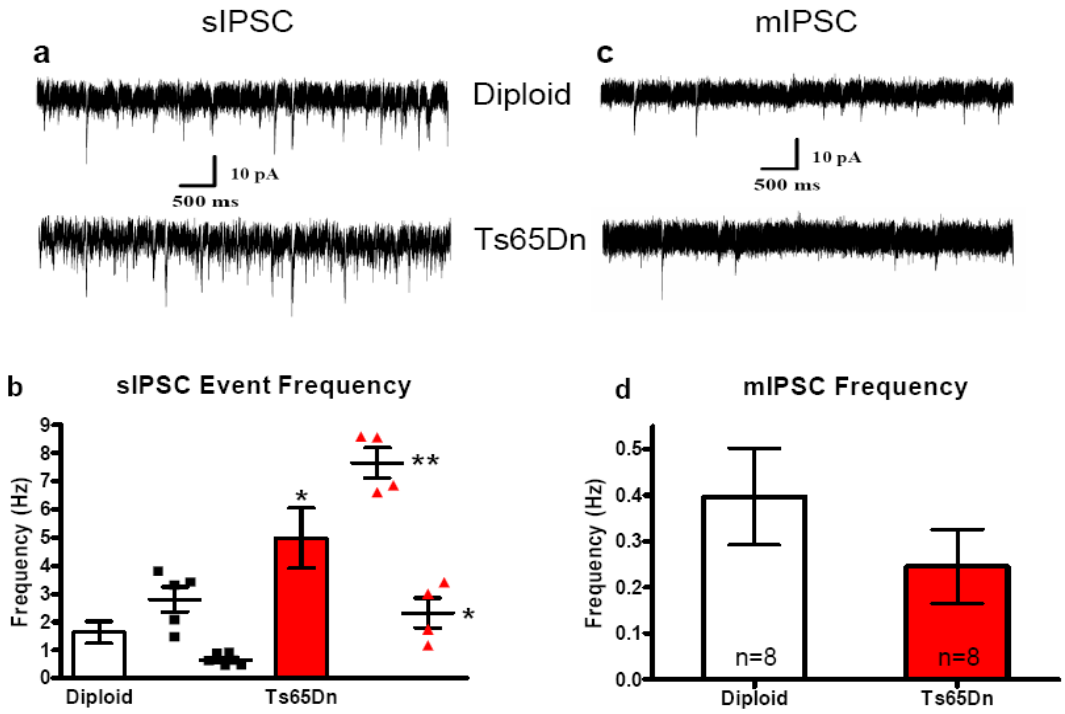


Figure 6. Spontaneous IPSCs are ~3 fold more frequent in Ts65Dn than diploid CA1 pyramidal neurons.

A. Traces from diploid and Ts65Dn CA1 pyramidal neurons demonstrating the relative increase in sIPSC frequency.

B. In Ts65Dn (n=8), the frequency of sIPSCs is significantly higher than in diploid (n=11, *p<0.005; bar graphs). Scatter plots show each frequency value from individual neurons cluster at high and low frequencies. Both the fast and slow cluster averages are significantly faster in Ts65Dn (n=4, 4) respectively than diploid (n=5, 6; **p<0.0005, *p<0.005).

C. Traces showing miniature IPSCs of diploid and Ts65Dn CA1 pyramidal neurons.

D. Averaged mIPSC frequencies were not significantly different between Ts65Dn and diploid

Figure 7

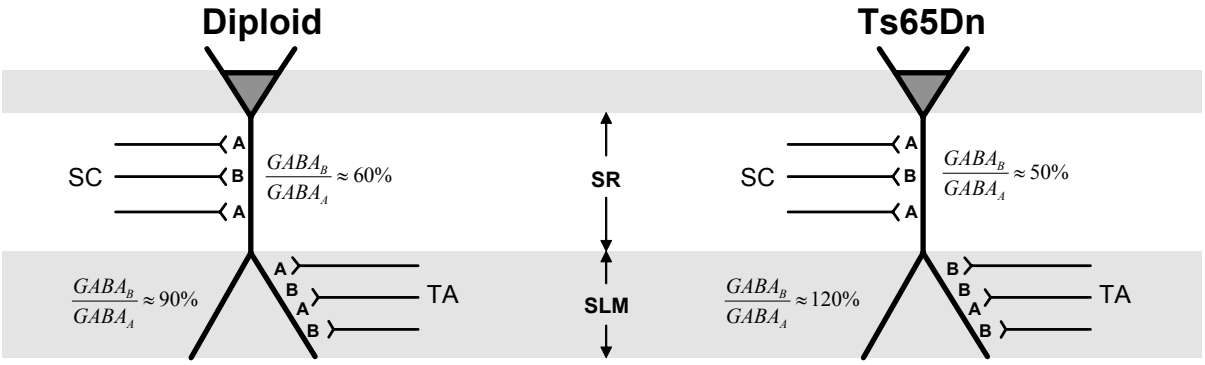


Figure 7. Model for dysfunctional hippocampal inhibitory circuitry in Ts65Dn mouse.
Depicts the relative magnitude of Schaffer collateral (SC) and temporoammonic (TA) inhibitory inputs to diploid and Ts65Dn CA1 pyramidal neurons within the SR and SLM respectively. In diploid and Ts65Dn CA1 neurons $GABA_B$ input is approximately half $GABA_A$ input in the SR. In contrast, the $GABA_B$ input in the SLM increases from about *90% of the $GABA_A$ input in diploid to *120% in Ts65Dn CA1 neurons. (*approximate values from overall $GABA_B/GABA_A$ ratio, see inset Figure 3a).

Table 1. Spontaneous and miniature IPSCs

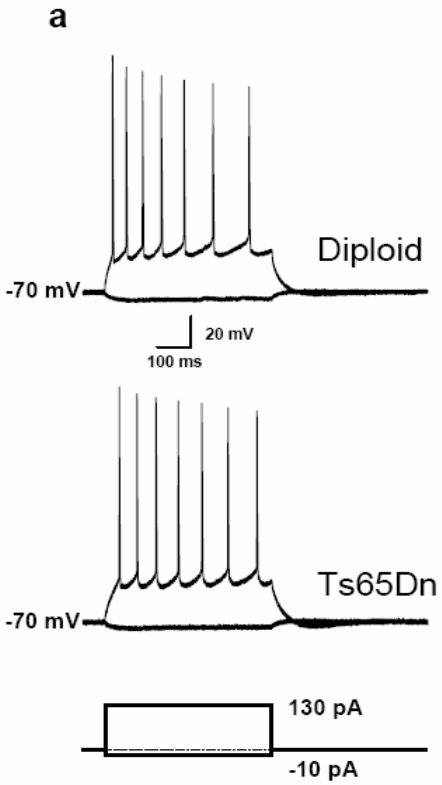
	Amplitude (pA)	Decay Time (ms)	Rise Time (ms)	Frequency (Hz)	Amplitude (pA)	Decay Time (ms)	Rise Time (ms)	Frequency (Hz)
sIPSC	**Diploid** (n=11)				**Ts65Dn** (n=8)			
normal ACSF	13.34 ± 1.02	11.30 ± 0.90	1.15 ± 0.02	1.63 ± 0.39	13.89 ± 0.91	11.35 ± 0.78	1.27* ± 0.05	4.99* ± 1.06
	Diploid (n=7)				**Ts65Dn** (n=6)			
high K+ ACSF	15.15 ± 1.14	11.99 ± 0.84	1.15 ± 0.07	9.39 ± 2.34	15.15 ± 1.98	12.20 ± 1.57	1.33 ± 0.09	10.64 ± 2.09
	Diploid (n=8)				**Ts65Dn** (n=8)			
mIPSC	8.67 ± 0.32	9.15 ± 0.41	1.12 ± 0.05	0.39 ± 0.11	9.14 ± 0.60	9.25 ± 0.66	0.97 ± 0.06	0.246 ± 0.08

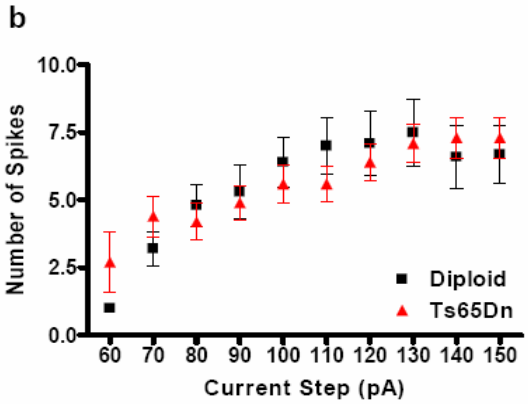
Table 2. Passive membrane properties relate to Girk2 gene dosa

	Cm (pF)	**Rm** (MΩ)	**Vm** (mV)
Diploid/GIRK2$^{(+/-)}$ (n=23)	36.77 ± 2.45	67.04 ± 4.28	-61.7 ± 0.7
Diploid/GIRK2$^{(+/+)}$ (n=73)	33.22 ± 0.90	57.80 ± 1.54	-62.2 ± 0.4
Ts65Dn/GIRK2$^{(++/-)}$ (n=6)	27.27 ± 1.55	67.27 ± 9.49	-63.1 ± 1.2
Ts65Dn/GIRK2$^{(++/+)}$ (n=39)	25.77 ± 0.75	59.06 ± 2.40	-64.4 ± 0.5
ANOVA	P<0.0001	P=0.08	P<0.005
Post - hoc trend test	P<0.0001	N/A	P<0.005

Membrane capacitance (Cm), membrane resistance (Rm), resting membrane potential (Vm).

Supplemental Figure 1





Supplemental Figure 1. Normal intrinsic excitability of CA1 Ts65Dn pyramidal neurons

A. Representative examples of voltage responses to -10 and 130 pA current injection in current-clamp for diploid and Ts65Dn neurons.

B. Normal spike frequency-dependence for suprathreshold current injection; diploid (n=23) and Ts65Dn (n=28) spike rate at increasing current steps.

Supplemental Table 1. Spike properties in diploid and Ts65Dn CA1 pyramidal neurons

	Diploid (n=23)	**Ts65Dn** (n=28)
Max Rise Slope (pA/ms)	270.2 ± 13.6	254.4 ± 8.4
Peak Amp (mV)	122.8 ± 1.5	122.6 ± 0.8
Half-width (ms)	1.6 ± 0.03	1.6 ± 0.02
Threshold (mV)	-44.7 ± 0.9	-43.7 ± 0.6

(1st spike of each neuron)

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CHAPTER 4

DISCUSSION

In this work I evaluated some of the functional consequences of overexpression of the G-protein coupled inward rectifying potassium channel subunit 2 (GIRK2) in the hippocampus of Ts65Dn, a mouse model of Down syndrome (DS). GIRK2 overexpression is complemented by overexpression of another subunit, GIRK1, which together comprise GIRK channels in the hippocampus (Harashima et al., 2006). Since GIRK2 overexpression drives overexpression of GIRK1 subunits an increased expression of functional GIRK channels is likely in Ts65Dn. I therefore **hypothesized that the three copies of the *Girk2* gene in DS drive functional overexpression of GIRK channels which results in dysfunctional inhibitory transmission within the Ts65Dn mouse hippocampus**. I evaluated this hypothesis with the accomplishment of the aims stated in the introduction. Briefly:

Specific Aim #1: To determine whether GIRK current in hippocampal neurons is dependent on gene dosage in the Ts65Dn DS mouse model. Hippocampal neurons cultured from Ts65Dn mice showed a significant increase in GIRK current density under basal and high K^+ experimental conditions (Figure 2, Chapter 2) with no evident change in coupling mechanisms (Figure 5, Chapter 2). Stationary fluctuation analysis of GIRK currents indicated that the change in current density was not a result of changes in channel properties but an increase in the density of functional GIRK channels at the plasma membrane (Figure 4 and Table 2, Chapter 2). Furthermore, CA1 pyramidal neurons from acute hippocampal slices showed significant increases in the ratio of

synaptically induced GIRK currents to pure $GABA_A$ currents (Figure 2, Chapter 3). Additionally, this GIRK/$GABA_A$ ratio was significantly dependent on *Girk2* gene dosage (Figure 3, chapter 3). Thus the extra *Girk2* gene copy leads to an increase in GIRK channel function in Ts65Dn hippocampus.

Specific Aim #2: To establish the contribution of GIRK2 containing channel activity to inhibitory GABAergic post-synaptic currents (IPSCs) in the Ts65Dn DS mouse model. GIRK channels contribute to approximately 50% the $GABA_A$ mediated inhibition at proximal CA1 synapses within the stratum radiatum for both diploid and Ts65Dn hippocampus. Whereas, at distal CA1 synapses within the stratum lacunosum-moleculare, $GABA_B$ mediated GIRK currents were approximately 90% of the $GABA_A$ mediated inhibition in diploid hippocampus, but in Ts65Dn, $GABA_B$ mediated GIRK currents were significantly increased to 120% of the $GABA_A$ mediated inhibition (Figure 2 and 7, Chapter 3). Therefore the increased GIRK channel function in Ts65Dn is input specific and primarily affects distal CA1 synapses.

Specific Aim #3: To evaluate the impact that GIRK2 containing channels have on signal integration and hippocampal circuitry in the Ts65Dn DS mouse model. The dynamic range of $GABA_B$ and $GABA_A$ synaptic inhibition was significantly reduced in Ts65Dn CA1 pyramidal neurons at stratum lacunosum-moleculare synapses but not within the stratum radiatum (Figure 4, chapter 3). Short-term facilitation of digitally isolated $GABA_A$ergic currents was decreased only at stratum radiatum synapses in Ts65Dn hippocampi (Figure 5, Chapter 3). Hence, Ts65Dn hippocampi have disrupted

integration and circuit imbalance of inhibitory transmission associated with GIRK channel functional overexpression.

Effect on Intrinsic Neuronal Properties

Girk2 gene dosage strongly influences passive intrinsic properties of hippocampal neurons. Cell surface area (based on membrane capacitance) is inversely related to the *Girk2* gene number (Figure 1a; Table 2, Chapter 3). Ts65Dn neurons have smaller surface area than diploid which can indicate a less extensive dendritic arbor. Decreases in dendritic length can promote summation of excitatory and inhibitory postsynaptic potentials (E-, IPSP) to an extent that Ts65Dn neurons are more susceptible to changes in

Figure 1

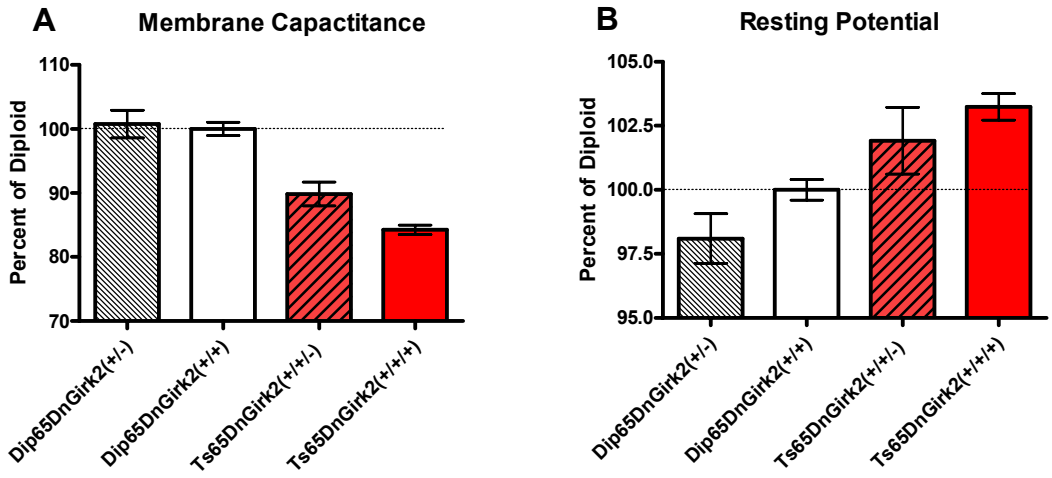

Figure 1. Girk2 gene dosage effect on passive membrane properties. Estimates of cell surface area, based on membrane capacitance (A), are significantly dependent on Girk2 gene dosage (ANOVA $p<0.0001$, post hoc trend test $p<0.0001$). Likewise resting membrane potentials (B) show significant dependence on the number of Girk2 gene copies (ANOVA $p<0.005$, post hoc trend test $p<0.005$). Membrane capacitance and resting membrane potentials are normalized to the averaged value for Dip65DnGirk2$^{(+/+)}$.

inhibitory or excitatory inputs than diploid counterparts. As such, Ts65Dn neurons would have a greater dynamic range in their homeostatic activity than diploid neurons and this may compromise the efficacy of plasticity mechanisms. Imbalanced homeostasis may thus compromise synaptic plasticity and contribute to the cognitive deficits associated with DS.

Questions remain for future studies as to whether GIRK expression can disrupt the architecture of individual neurons apart from cell surface area. Whether the deficits in dendritic branching of Ts65Dn cortical pyramidal neurons (Dierssen et al., 2003) correlates with GIRK2 expression or is related to other genes on the Ts65Dn chromosome has not been investigated. Dendritic branching could be affected by GIRK expression and activity levels since branching patterns are dependent on intrinsic and extrinsic factors (Libersat, 2005).

Girk2 gene number also influences resting membrane potential of hippocampal neurons. An increase in GIRK2 expression hyperpolarizes neurons, whereas, GIRK2 elimination depolarizes (Figure 1b; Table 2, Chapter 3; Figure 1, Appendix B) (Luscher et al., 1997; Koyrakh et al., 2005). These data were collected through whole-cell recordings at somatic locations where GIRK channel expression is comparatively low compared to the dendritic arbor. Where the surface to volume ratio is much greater, as in the dendrites, GIRK2 influence on resting membrane potential differences is likely much more pronounced (Chen and Johnston, 2005). Whole-cell recordings from dendrites of Ts65Dn or other mouse models of DS have not been performed but are warranted. Changes in dendrite membrane potentials can have far reaching effects. Chronic depolarization alters dendritic branching and spine density of neurons in culture (Sohya et al., 2007). Synaptogenesis may be impacted since pruning and maturation of synapses

has been shown to be dependent on depolarizing activity (Hubel et al., 1977). Integration of signals by dendrites is strongly influenced by resting membrane potentials of the dendrites themeselves (Gasparini and Magee, 2006) which can subsequently impact synaptic plasticity. Hyperpolarized synapses need more excitatory input to release the voltage-dependent Mg^{2+} block on NMDA receptors (Collingridge et al., 1988) thus impeding NMDA receptor-dependent plasticity mechanisms.

Effect on Neuronal Viability

Evidence indicates that CNS dysfunction in DS and other cognitive disorders are correlated with decreased neuronal viability. Reduced absolute brain volume reduction is characteristic of DS individuals with particular deficits in cerebellar and other brain regions (Pinter et al., 2001). Questions arise as to whether the decrease in cerebral volume is embryological and developmental or a result of later problems. Delays in prenatal growth of the Ts65Dn cerebral cortex and hippocampus due to longer cell-cycle duration and reduced neurogenesis causing hypocellularity and impaired synaptic development of Ts65Dn neocortex after birth have been recently reported, which suggests that embryonic deficits may underlie some postnatal disabilities in Ts65Dn (Contestabile et al., 2007). Neurons cultured from both DS patients and model animals are also reportedly more vulnerable to apoptosis (Sawa, 1999). A majority of research investigating cell death in DS has been limited to several apoptosis-related genes, including those related to oxidative stress, and transcription factors overexpressed on chromosome 21 (Sawa, 1999; Wolvetang et al., 2003; Porta et al., 2007). Recent data suggest that GIRK channel activity may also play a role in neuronal viability. Chronic treatment with fluoxetine (Prozac), a selective serotonin reuptake inhibitor and antagonist

of GIRK channels (Kobayashi et al., 2003) at clinically relevant doses, rescues deficits in neurogenesis in Ts65Dn mice (Clark et al., 2006). Interestingly mice with a GIRK2 pore mutation (*weaver* mice) show remarkably diminished neuronal populations in CNS regions that rely heavily on GIRK2 channel function (Harkins and Fox, 2002). Fluoxetine treatment also rescued the motor deficits and suppressed neuronal death in *weaver* mouse cerebellum and pontine nuclei, as well as effectively blocking the *weaver* mutant GIRK channel (Takahashi et al., 2006). These findings are strong indications that GIRK channel activity can influence the viability of neuronal populations and CNS function.

Effect on Structural and Functional Circuitry

GABAergic transmission shapes circuitry during development and influences excitatory and inhibitory synaptogenesis (Akerman and Cline, 2007). With an increase in slow inhibitory currents in Ts65Dn at a relatively early developmental age (10-20 days in culture from postnatal day 1-2 animals; or slices from 2-3 week old animals; Figure 2, Chapter 2; Figure 2, Chapter 3), and the genetic influence since gamete formation, the impact that disrupted inhibition may have on neuronal circuitry is profound. In fact, at early ages defects in circuit architecture have been identified in DS individuals. Deficits in dendritic spine density, synapses, shorter basilar dendrites, altered morphology and defective cortical layering and patterns of lamination have been identified in DS infants and newborns (Takashima et al., 1981; Golden and Hyman, 1994; Weitzdoerfer et al., 2001). Dendritic branching in DS infants (<6 mo) was greater than normal but declined with age so that by 2 years branching was significantly less than in normal children (Becker et al., 1986). Similar dendritic malformations have been identified in adult Ts65Dn mice. Ts65Dn hippocampi show significantly smaller ratio of synapse to neuron

compared to diploid controls, an effect that is predominantly due to decreases in asymmetric (presumably excitatory) synapses (Ayberk Kurt et al., 2004). Layer III pyramidal neurons from Ts65Dn cortex are smaller, less branched and demonstrate a marked reduction in spine density than diploid neurons (Dierssen et al., 2003). Deficits in spine density also have been confirmed in other brain regions including fascia dentata, motor, somatosensory and entorhinal cortices (Belichenko et al., 2004). The architectural malformations may be independent of the disrupted inhibition and could be due to other genes or gene combinations from the triplicated chromosome such as Tiam1 or Dscr1 (see appendix: Best et. al. 2006). However, neurite outgrowth is disrupted by exogenous *weaver* (wv) mutant *Girk2*^{wv} suggesting a more direct role of GIRK channel function in circuit formation (Schein et al., 2005).

In addition to structural influences, the increased functional GIRK responses in Ts65Dn neurons, likely has a great impact on information processing of neuronal circuitry. Hippocampal GIRK channel expression patterns complement the data demonstrating increased functional responses in distal CA1 dendrites of the stratum lacunosum-moleculare (SLM) compared to proximal dendrites of the stratum radiatum (SR) (Figure 2, Chapter 3) (Liao et al., 1996; Drake et al., 1997). Layer III neurons of the entorhinal cortex project directly to CA1 pyramidal neurons in the SLM via the temporoammonic (TA) pathway (Steward and Scoville, 1976). Stimulation within the SLM of TA fibers has been shown to block CA1 pyramidal neuron spikes driven by SR stimuli (Dvorak-Carbone and Schuman, 1999). This spike blocking property of TA stimulation is dependent on $GABA_B$ stimulation and is most efficacious when timed with the peak of hyperpolarizing current (Dvorak-Carbone and Schuman, 1999) indicative of the contribution of GIRK channels in shunting excitatory input. Interestingly, the relative

metaplastic state of synapses in the TA termination zone (i.e. SLM) demonstrated variable efficacy in blocking Schaffer collateral (SC) driven spikes. When synapses in the SLM were potentiated by an LTP inducing protocol, the ability to block SC driven spikes was enhanced, likewise, LTD protocols in the SLM decreased the efficacy to block spikes (Remondes and Schuman, 2002). Furthermore, stimuli of the TA path was also able to impede the potentiation of SC-CA1 synapses by LTP protocols and, remarkably, this LTP impediment was eliminated by $GABA_A$ block (Remondes and Schuman, 2002). This return to normal LTP levels after TA inhibitory impact, is reminiscent of the LTP deficits in Ts65Dn, which are also eliminated with $GABA_A$ block (Kleschevnikov et al., 2004; Costa and Grybko, 2005; Fernandez et al., 2007). Could similar mechanisms involving TA inputs be at play in the diminished plasticity of Ts65Dn synapses? There is a subset of hippocampal interneurons whose projections are restricted to CA1 pyramidal neurons dendrites within the SLM (Freund and Buzsaki, 1996). These stratum oriens-alveus interneurons (OAI) receive collateral input from the CA1 pyramidal neurons themselves and thus feedforward inhibition of the CA1 pyramidal neurons is generated (Samulack and Lacaille, 1993). The strength of feedforward inhibition by the OAIs is dependent on the potentiated state of the CA1 neurons themselves and can regulate the direct entorhinal input to CA1 pyramidal neurons via the TA path (Figure 2) (Maccaferri and McBain, 1995). When CA1 pyramidal neurons are potentiated they increase stimulation to OAIs and subsequently strengthen the inhibition within the SLM. In contrast, depression of CA1 pyramidal neurons diminishes the strength of inhibition at SLM synapses. In this way the relative influence that TA afferents have on CA1 output is regulated by feedforward inhibition through OAIs at SLM synapses. The synaptic weight of TA inputs could then be modified by the increase in $GABA_B$ergic function in Ts65Dn.

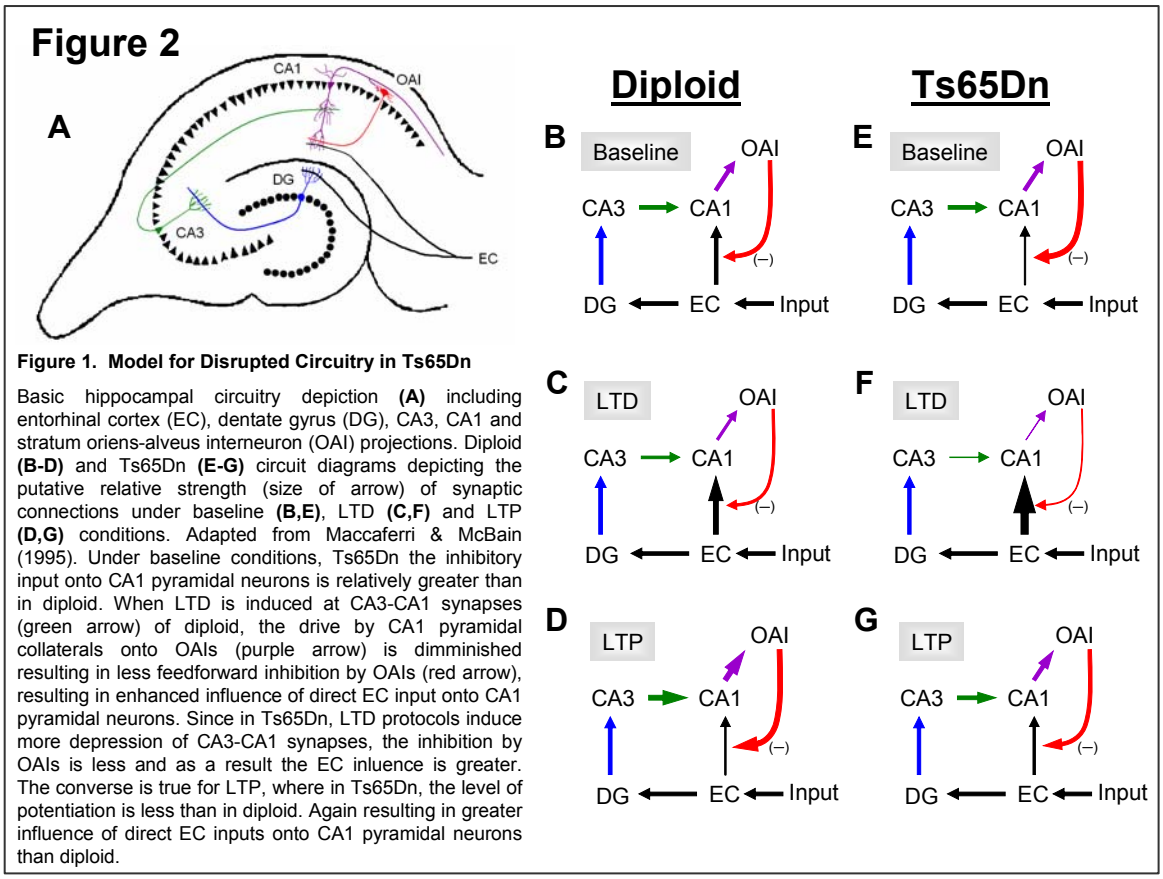

Figure 1. Model for Disrupted Circuitry in Ts65Dn

Basic hippocampal circuitry depiction **(A)** including entorhinal cortex (EC), dentate gyrus (DG), CA3, CA1 and stratum oriens-alveus interneuron (OAI) projections. Diploid **(B-D)** and Ts65Dn **(E-G)** circuit diagrams depicting the putative relative strength (size of arrow) of synaptic connections under baseline **(B,E)**, LTD **(C,F)** and LTP **(D,G)** conditions. Adapted from Maccaferri & McBain (1995). Under baseline conditions, Ts65Dn the inhibitory input onto CA1 pyramidal neurons is relatively greater than in diploid. When LTD is induced at CA3-CA1 synapses (green arrow) of diploid, the drive by CA1 pyramidal collaterals onto OAIs (purple arrow) is dimminished resulting in less feedforward inhibition by OAIs (red arrow), resulting in enhanced influence of direct EC input onto CA1 pyramidal neurons. Since in Ts65Dn, LTD protocols induce more depression of CA3-CA1 synapses, the inhibition by OAIs is less and as a result the EC inluence is greater. The converse is true for LTP, where in Ts65Dn, the level of potentiation is less than in diploid. Again resulting in greater influence of direct EC inputs onto CA1 pyramidal neurons than diploid.

Further experiments involving TA modulation of SC-CA1 plasticity with agents that block $GABA_B$ or GIRK channel activity would be particularly intriguing (such as tertiapin for GIRK channels and CGP55845 for $GABA_B$ receptors); especially so when considering an increase in $GABA_B$/GIRK inhibition, as well as the diminished LTP and enhanced LTD of Ts65Dn mice (Siarey et al., 1997; Siarey et al., 1999). The regulation of feedforward inhibition may be disrupted in Ts65Dn hippocampus, and similarly timing and the relative strength of inputs between TA and SC fibers would be disrupted such that hippocampal plasticity mechanisms are compromised.

It is evident that the relative timing in pre- and postsynaptic neuron activity can determine the strength of synaptic connections (Markram et al., 1997; Bi and Poo, 1998). The ability of SLM excitatory synapses to induce CA1 pyramidal cell spiking is dependent on coincident timing of excitatory SC inputs (Jarsky et al., 2005).

Furthermore, in order for TA generated EPSPs to reach CA1 pyramidal somas, SC input must be phase shifted by one-half theta frequency (Ang et al., 2005). This coincident timing serves as a gate by which CA1 pyramidal neurons activity is regulated and can modulate activity-dependent plasticity. An increase in $GABA_B$ activity has been demonstrated to decrease the oscillation frequencies of interneuron networks (Scanziani, 2000). This increased functionality of $GABA_B$/GIRK2 complexes in Ts65Dn, may therefore, slow network activity and disrupt timing such that coordinated modulation of this coincidence gate is dysfunctional (Figure 3). GIRK channels are effectors of multiple neurotransmitter receptors in addition to $GABA_B$ receptors, therefore these neurotransmitters may modulate GIRK-mediated coincidence gate and either enhance or attenuate various forms of synaptic plasticity in Ts65Dn (Goh and Pennefather, 1989; Staubli and Xu, 1995; Scheiderer et al., 2004; Li et al., 2007).

Following this idea, the multiplicity of GIRK channel activators suggests that disparate neurotransmission converges on the GIRK channel which serves as a common effector for such signaling. Hippocampal neurons receive extrahippocampal inputs from serotonergic, dopaminergic, cholinergic, neuropeptide Y and other neurotransmitter systems (Andersen, 2007). All of which can activate GIRK channels. Questions arise when considering multiple agonists on GIRK channels. Do these GIRK agonists share a common pool of GIRK channel effectors, or does each neurotransmitter sequester its own set of GIRK channels within postsynaptic microdomains? Simultaneous application of GIRK agonists suggest that different receptors share a common pool of GIRK channels and that the quantity of GIRK channels themselves is the limiting factor in response to the activity of these neurotransmitters (Sodickson and Bean, 1998). That is to say, under maximal stimulation, one neurotransmitter occludes the effect of a second

Figure 3

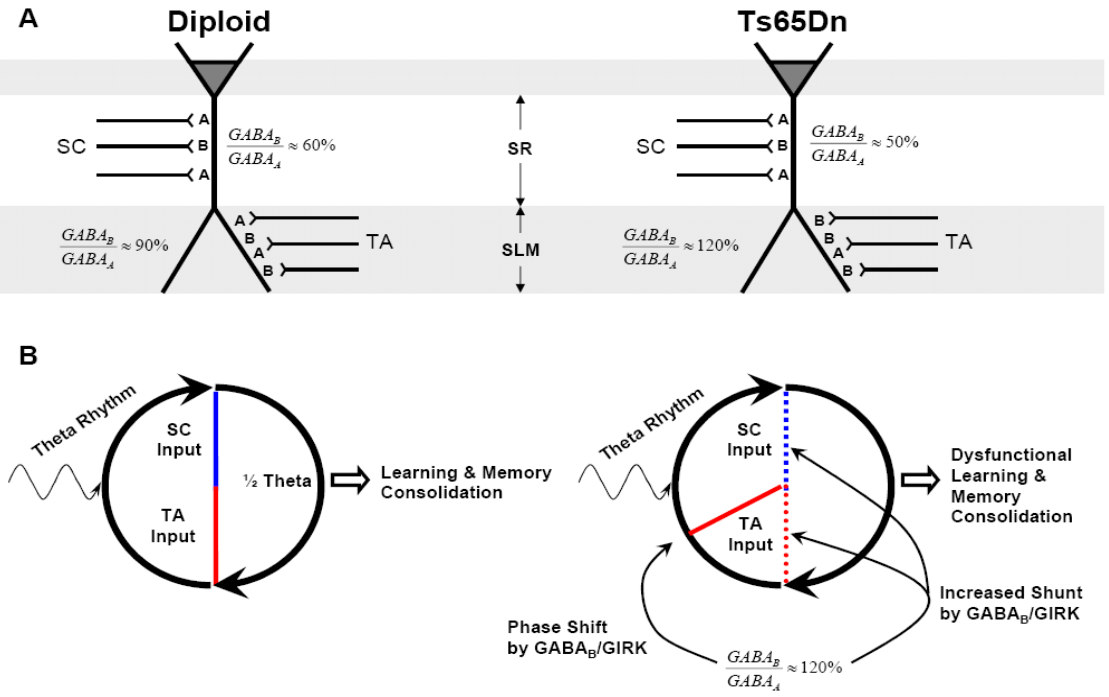

Figure 3. Model for dysfunctional hippocampal inhibitory circuitry in Ts65Dn.
A. Depicts the relative magnitude of Schaffer collateral (SC) and temporoammonic (TA) inhibitory inputs to diploid and Ts65Dn CA1 pyramidal neurons within the SR and SLM respectively. In diploid and Ts65Dn $GABA_B$ input is approximately half $GABA_A$ input in the SR. In contrast, the $GABA_B$ input in the SLM increases from about 90% of the $GABA_A$ input in diploid to 120% in Ts65Dn.
B. A figurative illustration of the balance in timing of SC and TA inputs for diploid which may be disrupted in Ts65Dn. Theta rhythms and other hippocampal network oscillations correlate with learning and memory consolidation. SC inputs must be phase shifted by one-half theta frequency for TA generated EPSPs to reach CA1 pyramidal somas (Ang et al., 2005). Furthermore, the strength and timing of Schaffer collateral inputs determines whether TA initiated CA1 dendritic spikes propagate beyond the SLM and initiate firing (Jarsky et al., 2005). In Ts65Dn, an increase in $GABA_B$ function can slow interneuron oscillations (Scanziani 2000) disrupting the timing between SC and TA inputs. It can also shunt SC induced CA1 activity (Dvorak-Carbone and Schuman 1999). The result of these disruptions is dysfunctional consolidation of learning and memory.

neurotransmitter when activating GIRK channels. By contrast, submaximal agonist levels induce additive or even supra-additive effects by increasing the quantity of active G-proteins signaling GIRK channels to open. Thus GIRK channels act as coincidence detectors where limits in the upper range of activation are set by channel expression. Therefore in Ts65Dn mice, where GIRK channels are overexpressed, the balance

between additive and occlusive effects is disrupted in such a way that simultaneous signaling by multiple neurotransmitters is less regulated and precision is lost. Synaptic plasticity and homeostatic regulation may be compromised in Ts65Dn as a consequence of this loss in synchronization of neuronal circuits.

Impact on Down Syndrome

The quality of life for Down syndrome individuals has improved immensely in recent years. People with DS have been able to take advantage of educational and occupational opportunities that enable them to live better, more productive lives. Better understanding of developmental progress and insights into intervention programs have provided means whereby caregivers can propel DS individuals to achieve levels of functioning never before thought possible. The best educational, developmental and life interventions can only go so far to improve cognitive deficits and improve mental functioning in DS. In order to improve cognitive function beyond what intervention programs can do, it is indispensable first to understand the basis of CNS impairments in DS and then try to reverse these dysfunctions with more effective pharmaceutical or genetics tools.

Recent studies have shown that pharmacological intervention at $GABA_A$ receptors may, in part, treat CNS dysfunction in DS to the point where normal physiological function is achieved under certain conditions (Kleschevnikov et al., 2004; Costa and Grybko, 2005; Fernandez et al., 2007). The results from these studies are promising in that simple blockade of inhibitory neural transmission can improve performance. However, the mechanism by which $GABA_A$ block improves function is not understood, nor is there a clear relationship between extra gene copies and $GABA_A$ergic dysfunction

in DS (genes encoding $GABA_A$ receptor subunits are not on human chromosome (Hsa.) 21 but on 4, 5, 15 and X human chromosomes (Russek, 1999)). The work presented in this thesis however, for the first time sheds light and builds rational why GABAergic transmission is disrupted in Ts65Dn mice and relates it directly to a gene found on Hsa.21, *Girk2*. I demonstrate that there is an increase in $GABA_B$ergic function mediated by GIRK channels and that this disrupts the balance between $GABA_A$ and $GABA_B$ mediated signaling. This imbalance can shift tonic GABAergic transmission so that application of $GABA_A$ antagonists through a homeostatic mechanism can improve function to that resembling diploid. Therefore agents (e.g. GIRK channel blockers) that also shift this inhibitory balance between $GABA_A$ and $GABA_B$ may be new promising tools to improve cognitive function in DS.

Summary

In short, we have found that the Ts65Dn, mouse model of DS, overespresses GIRK channels as a result of an extra *Girk2* gene. This overexpression is functional in nature, in that $GABA_B$-induced GIRK currents are elevated in Ts65Dn hippocampal neurons. The elevated GIRK channel function increases inhibitory potassium conductance that can disrupt excitatory drive of direct entorhinal input into hippocampus and create timing desynchronization in the hippocampal circuitry. These disruptions, if extrapolated to humans, likely contribute to the mental disabilities of DS individuals and as such would be promising targets for therapeutic interventions.

FUTURE

An important and far reaching goal in studies of DS mice is to design rational therapy that may be used to alleviate certain neurological phenotypes. Such an approach has been recently successful with an adult mouse model of neurofibromatosis (NF1). NF1 is a neurological disorder caused by mutations in the gene encoding neurofibromin, where increases in p21Ras activity is a key biochemical marker related to the pathophysiology in both human and mouse model (Li et al., 2005). Pharmacological treatment reversed the biochemical, electrophysiological, and cognitive deficits in an adult mouse model of NF1. This provides hope that a potential treatment of cognitive impairments in people with NF1 is possible (Li et al., 2005). Although the genetic picture of DS is more complicated that NF1 and its strong developmental component creates additional challenges, a pharmacological goal to ameliorate some of the phenotypes of DS is an important target and hope for DS patients and their families. GIRK channel overexpression has been a pharmaceutical target for treating dysfunctional neurogenesis in DS mouse models (Clark et al., 2006), and therefore a promising future target for DS individuals.

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Appendix

ABNORMAL SYNAPTIC PROPERTIES IN DOWN SYNDROME: LESSONS FROM MOUSE MODELS

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OVERVIEW

With the advent of mouse models of Down syndrome (DS) the possibilities to further explore and understand the dysfunctions associated with DS expands. In particular, the basic and cellular neurophysiology can now be achieved in mice, which was not possible in human DS. Such that specific DS dysfunctions can now be separated from the overall disorder and examined in a manner which may provide interventions that directly address particular abnormalities. The function of well defined regions and even certain genes within the triplicated chromosome are being understood with greater clarity to the extent that some DS specific phenotypes could be therapeutically ameliorated in the future. For example, the phenotype in a neurofibromatosis mouse model was reversed with pharmacological treatment (Li et al., 2005).

INTRODUCTION

Mental retardation can be attributed to improper neural functioning caused by a broad spectrum of abnormalities that disrupt central nervous system (CNS) function. The cognitive deficits associated with DS are the result of expression of extra gene copies from chromosome (Chr.) 21 affecting CNS function. Mental retardation in DS is characterized by memory deficits, developmental delays, and other cognitive abnormalities. Neurophysiological information is needed to understand the mechanisms causing the mental retardation in DS. Cognitive impairment is one of three phenotypes, including early onset of Alzheimer disease neuropathology and muscle hypotonia, that affect nearly all DS individuals.

In 1866 the English physician, John Langdon Down, first illustrated the characteristic features of individuals, which we now associate with DS (Down, 1866). In 1959, the French geneticist, Jerome Lejeune, showed that DS is caused by chromosomal abnormalities related to the presence of an extra whole or part of Chr. 21, which was termed trisomy 21 (Ts21) (Lejeune, 1959; Antonarakis et al., 1985; Korenberg et al., 1990). There are more than 200 known genes on Chr. 21 (Hattori et al., 2000), which can contribute to the numerous DS phenotypic abnormalities. A chromosomal critical region (DSCR) for many of the neurological features such as mental retardation has been hypothesized to be localized between the carbonyl reductase (CBR) and transcriptional regulator *ets*-related gene (ERG) loci (Delabar et al., 1993; Dahmane et al., 1995; Toyoda et al., 2002). However, genes outside the DSCR also can be involved in the DS phenotype (Korenberg et al., 1994) and recently the concept and existence of the DSCR has been challenged (Olson et al., 2004a).

Trisomy mouse models provide insight into the molecular and genetic effects that abnormal chromosome number has upon neurophysiological profiles. The distal segment of mouse Chr. 16 is homologous to nearly the entire long arm of human Chr. 21 (Figure 1). Therefore, mice with full or segmental trisomy 16 (Ts65Dn, Ts16Cje and Ts1Rhr) are

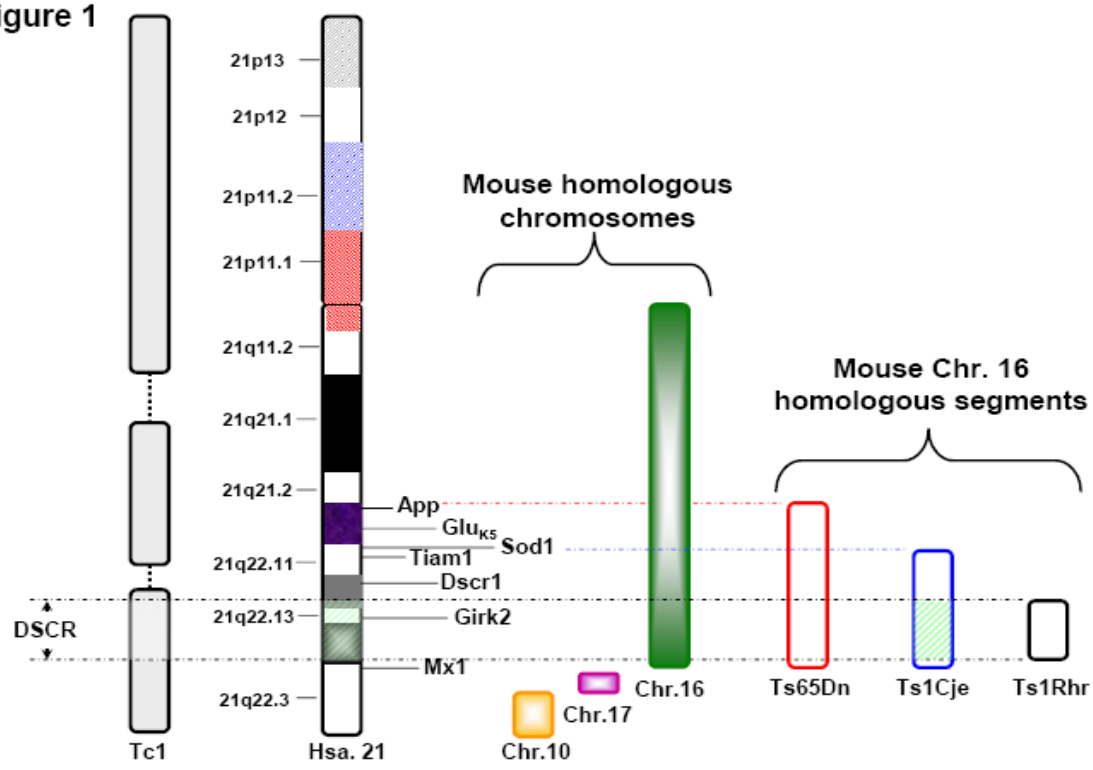

Figure 1. Human chromosome 21 (Hsa. 21) map showing the Down syndrome critical region (DSCR) and relationship of various genes to the trisomy mouse model chromosome segments. Homologous segments of mouse chromosomes 10, 16 and 17 are also represented corresponding to analogous portions of Hsa. 21.
Noted Genes: amyloid precursor protein (App), glutamate receptor subunit-5 kainate subtype (GluK5), superoxide dismutase-1 (Sod1), T-lymphoma invasion and metastasis-1(Tiam1), Down syndrome candidate region-1 (Dscr1), G-protein coupled inward rectifying potassium channel subunit-2 (Girk2), myxovirus (influenza virus) resistance-1 (Mx1).

considered genetic animal models of DS. Ts65Dn and Ts1Cje mice demonstrate impaired learning in spatial tests and abnormalities in hippocampal synaptic plasticity, which mirrors deficits seen in DS individuals. Mice in which only the DSCR is triplicated (Ts1Rhr) show no abnormalities related to craniofacial features (Olson et al., 2004a).

Recently an aneuploid mouse strain carrying ~92% of human Chr. 21 (Hsa21) has been developed (Tc1). Although chimeric for Hsa. 21 (Figure 1), these mice show many phenotypes consistent with DS and DS mouse models, such as spatial learning and memory deficits, abnormal synaptic plasticity, decrease in cerebellar granule cell populations, developmental heart problems and decreased mandibular size (O'Doherty et al., 2005). In all, these trisomic mouse models provide excellent tools by which to elucidate dysfunctions found in DS individuals.

We hypothesize that overexpression of genes from Chr. 21 disrupts homeostasis in the DS brain such that mechanisms underlying development, structure and plasticity of neuronal networks are compromised to the extent that the interaction between them and normal functioning is severely affected.

ABNORMAL SYNAPTIC PLASTICITY IN DS MOUSE MODELS

The hippocampus is part of the limbic system and plays an important role in learning and memory. It is a site for long-term synaptic plasticity that appears to be critical to memory formation, consolidation and retrieval and therefore has been extensively studied in the modeling of learning and memory. Brief high-frequency activation of specific inputs causes a persistent increase in synaptic responsiveness (an increase in the excitatory postsynaptic potentials termed long-term potentiation (LTP)) that under certain circumstances can last for hours, days or weeks (Bliss and Collingridge, 1993). We investigated the phenomenon of LTP in the CA1 region of hippocampi from Ts65Dn mice (Figure 2 insets) and found there to be reduced LTP over a period of 60 min compared to that of age-matched diploid-controls (Siarey et al., 1997).

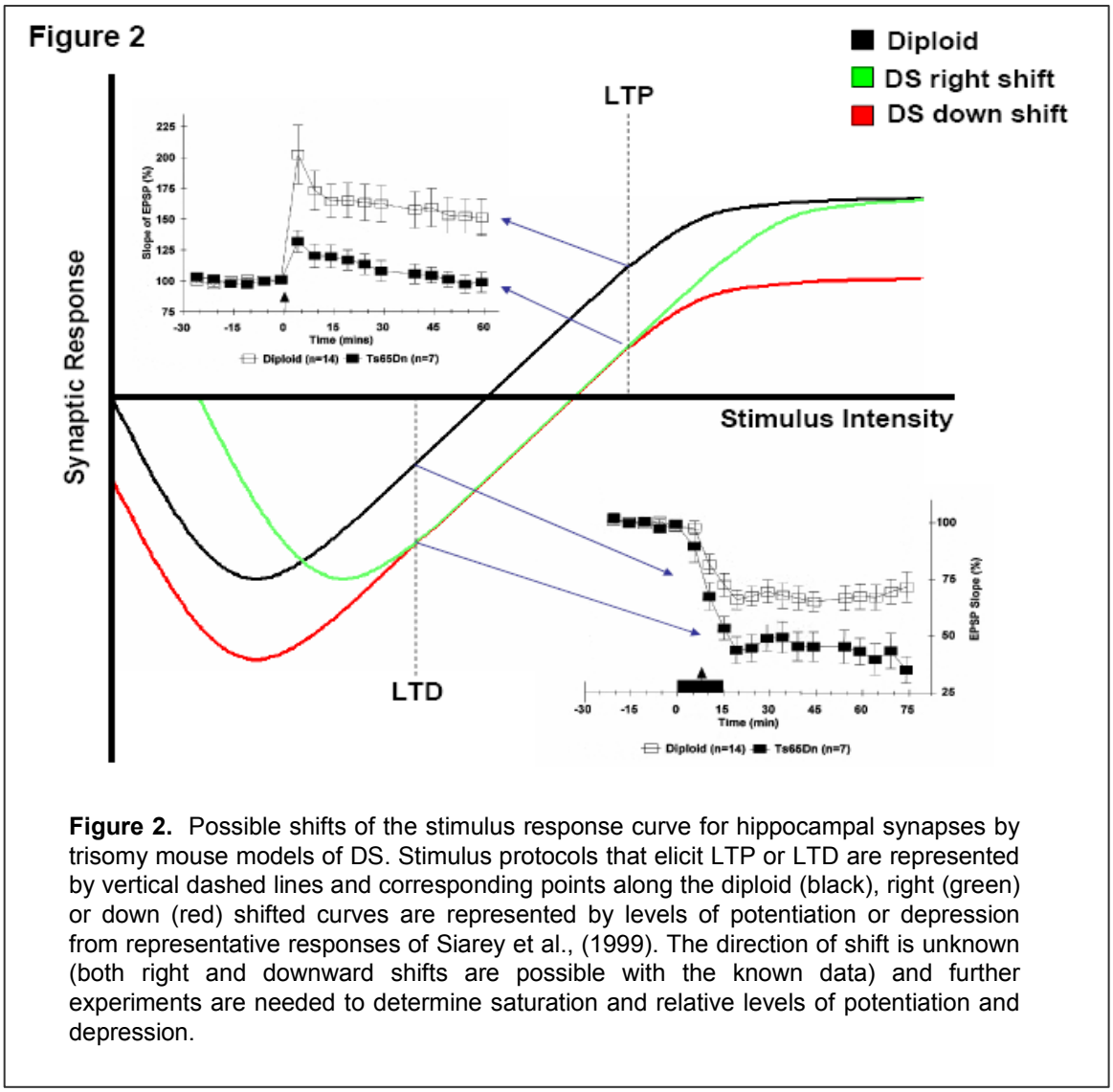

Figure 2. Possible shifts of the stimulus response curve for hippocampal synapses by trisomy mouse models of DS. Stimulus protocols that elicit LTP or LTD are represented by vertical dashed lines and corresponding points along the diploid (black), right (green) or down (red) shifted curves are represented by levels of potentiation or depression from representative responses of Siarey et al., (1999). The direction of shift is unknown (both right and downward shifts are possible with the known data) and further experiments are needed to determine saturation and relative levels of potentiation and depression.

No significant difference between Ts65Dn and diploid controls was revealed in a paired pulse protocol, suggesting that presynaptic plastic mechanisms are similar. In another set of experiments sequential LTP and long-term depression (LTD) were evoked from Ts65Dn hippocampi (Siarey et al., 1999). Both forms of use-dependent synaptic plasticity were abnormal in Ts65Dn compared to the diploid-controls; with LTP decreased, and LTD increased. We suggest that there may be a shift in the dynamic range of synaptic plasticity within the Ts65Dn hippocampus (Figure 2). These findings may explain the

reported spatial behavioral and learning impairments that are persistent throughout the life of the Ts65Dn mouse (Holtzman et al., 1996).

In recent months LTP from the Tc1 mouse dentate gyrus was investigated and also found to be impaired, while baseline and short term plasticity were unchanged (O'Doherty et al., 2005). These data parallel findings from the other DS mouse models. Behavioral tests of Tc1 mice correlate with the synaptic plasticity data, in that short-term memory was intact in the alternating T-maze, but long-term memory was impaired as evidenced by the novel-object recognition task. If mice that contain only triplicated DSCR (Ts1Rhr) reveal no changes in behavioral and physiological parameters then we may assume that compensatory mechanisms are sufficient to overcome the consequences of the extra chromosome segment restricted to the DSCR. Whereas, mice such as Tc1, Ts65Dn and Ts1Cje, which contain more than just the DSCR, are not capable of overcoming the consequences of the extra genetic material. This work is in progress.

Mechanisms that explain the deficits in LTP from these mice are unclear; however, recently deficits in LTP of Ts65Dn hippocampus were described by an increase in GABAergic tone. Deficits in LTP from the dentate gyrus of the Ts65Dn mouse were reversed when GABAergic inhibitory neurotransmission was blocked by 100 μM picrotoxin (Kleschevnikov et al., 2004). Likewise Ts65Dn dentate neurons showed an increased frequency of miniature inhibitory postsynaptic currents (mIPSCs) and a decrease in paired pulse facilitation (PPF), findings consistent with an increase in GABA release probability. In another study, 10 μM picrotoxin eliminated a deficit in theta burst stimulus (TBS) induced LTP in Ts65Dn CA1 hippocampal region (Costa and Grybko, 2005). These data suggest that an increased inhibitory tone explains the diminished LTP

of Ts65Dn hippocampus. Since basal excitatory synaptic transmission measured by extracellular recording electrodes appeared normal in these studies and in all of ours, we hypothesize that compensatory mechanisms to establish normal excitatory transmission do so at the expense of plasticity mechanisms.

GENETIC CONTRIBUTIONS TO ABNORMAL SYNAPTIC PLASTICITY

The contribution of the many trisomic genes to the deficits in synaptic plasticity remain unclear, however the use of DS mouse models that contain different (e.g. smaller) trisomic segments can clarify which genes are involved in the dysfunction. For example, the Ts1Cje mouse which contains a smaller triplicated segment than the Ts65Dn mouse (Figure 1). Studies examining the behavior of Ts1Cje mice reveal cognitive and behavioral abnormalities including low levels of locomotion, decrease in exploratory behavior, and impairment in spatial learning in the hidden platform and reverse hidden platform tasks in the Morris water maze {Sago 1998; Sago 2000}. Comparisons with the Ts65Dn mouse show that, although the Ts1Cje mouse had similar spatial learning deficits, these deficits were less severe than those found in the Ts65Dn mouse {Sago 2000}.

LTP and LTD in the isolated hippocampus of Ts1Cje mice are abnormal compared to diploid controls. LTP was reduced and LTD was augmented in comparison to diploid controls (Siarey et al., 2005) findings that parallel data from Ts65Dn hippocampus (Siarey et al., 1999). These changes are significant, but are less dramatic than were seen in Ts65Dn mice. Although the genetic backgrounds of Ts1Cje and Ts65Dn mice were similar, the two strains of mice were not produced in the same mating

scheme, as was the case in Sago et al. (2000). Therefore, direct comparisons of the relative changes between them must be interpreted very cautiously. Behavioral studies performed on Ts65Dn and Ts1Cje littermates (derived from the same mating scheme, thus carrying identical genetic background) demonstrated abnormalities in behavior, with the degree of impairment in Ts1Cje mice being more subtle than in Ts65Dn mice (Sago et al., 2000).

There is currently no reported evidence to link triplication of any single gene to a specific DS feature and it is unlikely that the overexpression of a single gene is responsible for the neurophysiological impairments in DS brain. Nevertheless, alterations in the expression levels of single genes can cause significant changes in phenotype, behavior and physiology as evidenced by the multitude of knock-out (knock-in) mice and the effects of the genetic manipulations. In order to characterize the effect of individual genes within the milieu of overexpressed genes in DS, the use of segmental trisomy mice crossed with knockout mice targeted for particular genes on the triplicated chromosomes segment would offer a strategy which can pinpoint the relationship of individual gene overexpression to those of the entire overexpressed segment. (This approach was attempted in regards to the App gene in Ts65Dn mice (Cataldo et al., 2003).)

There are a number of genes on the extra segment of Hsa. 21 and likewise mouse Chr. 16 that may be involved in abnormal plasticity in the DS mouse models, however, we will limit our discussion to the *App*, *Sod1,* Glu_{K5} and *Girk2* genes. Amyloid precursor protein (APP) is involved in the formation of amyloid plaques in Alzheimers disease and studies investigating the effects of App on LTP produced mixed results. An increase in LTP has been shown in a mouse that harbors a double human APP mutation (Jolas et al.,

2002); this is in contrast to a study with an APP-null mutant mouse that showed a decrease in LTP (Dawson et al., 1999). Interestingly the decrease in LTP in the APP-null mouse could be reversed in the absence of GABAergic inhibition (Fitzjohn et al., 2001).

Superoxide dismutase (SOD) is an important enzyme that regulates oxygen metabolism and levels of free radicals in the brain. Mice that overexpress SOD1 demonstrate impaired LTP and deficient spatial memory (Gahtan et al., 1998; Levkovitz et al., 1999; Thiels et al., 2000; Kamsler and Segal, 2003). Remarkably, blockade of $GABA_A$ activity rescued the LTP deficits similar to that seen in Ts65Dn mice (Levkovitz et al., 1999; Kleschevnikov et al., 2004; Costa and Grybko, 2005). Contrary to these reports, a more recent study found that SOD1 overexpression resulted in augmented LTP and in enhanced performance of spatial memory tasks (Spalloni et al., 2006).

Kainate receptors are a subtype of the excitatory glutamate receptor family. One subunit of these receptors, Glu_{K5} has been shown to be overexpressed and an increase in binding of the kainite receptor specific ligand [^{3}H](2*S*,4*R*)-4-methylglutamate ([^{3}H]SYM 2081) (A. KlineBurgess, 2001; Galdzicki and Siarey, 2003) has been demonstrated in the hippocampus of the Ts65Dn mouse. Kainate receptors have been reported to be expressed by hippocampal interneurons (Mulle et al., 2000) and their activity increases interneuron firing rates while paradoxically reducing GABA-mediated synaptic inhibition (Christensen et al., 2004; Maingret et al., 2005). Hence it is possible that over-expression of Glu_{K5} subunits contributes to an increase in GABAergic interneuron excitability but may reduce GABAergic inhibition in Ts65Dn mouse.

However we suggest that overexpression of *App* and *Glu_{K5}* genes alone cannot directly account for the neuroplasticity impairments in the Ts65Dn mouse, since they are

not on the Ts1Cje segment (see Figure 1). The Ts1Cje mouse is trisomic for a region of Chr. 16 that incorporates all the genes in the DSCR and spans between *Sod1* and *Znf295*, and thus lacks triplication of *App* and Glu_{K5} genes and overexpression of these gene products (Amano et al., 2004; Olson et al., 2004b). Synaptic impairments in Ts1Cje mouse should also not be attributed to Sod1 since there is a normal expression of *Sod1* in the brain of these mice (Sago et al., 1998; Amano et al., 2004). Therefore, since both the Ts65Dn and Ts1Cje mice show impaired synaptic plasticity, genes from within the common trisomic segment most likely cause the abnormal plasticity. One such gene could be *Girk2*. G-protein coupled inward rectifying potassium (GIRK) channels contribute to neuronal resting potential, excitability and firing properties. We have shown that GIRK2 protein is overexpressed ~1.5 fold in Ts65Dn hippocampus (Harashima et al., 2006) and is likely to be overexpressed in Ts1Cje as well. In GIRK2 knockout mice, LTP and LTD are also abnormal (A. Adeniji-Adele, 2004) but in the opposite direction from the DS mouse models, suggesting that the level of GIRK2 expression correlates to the level of potentiation or depression of hippocampal synapses.

Given the limited dynamic range of Schaffer-collateral/CA1 synapses (Savicc et al., 2003), a shift in the baseline synaptic strength could explain both a decrease in LTP and an increase in LTD for the Ts65Dn and Ts1Cje mouse models (Figure 2). The expression level of GIRK2 could effectively change the dynamic range of hippocampal synapses. Multiple G-protein coupled receptors, including the metabotropic $GABA_B$ receptor activate GIRK currents. GIRK channels have been shown to be highly expressed and constitutively active in CA1 pyramidal cell dendrites (Chen and Johnston, 2005; Koyrakh et al., 2005) and therefore likely to dramatically influence synaptic function

when overexpressed. $GABA_B$-mediated slow IPSCs are sufficient to inhibit NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) in dentate molecular layer interneurons (Mott et al., 1999). These slow IPSCs also block action potentials evoked by weak but not strong depolarizations (Mott et al., 1999). Furthermore, overexpression and activation of GIRK channels in cultured rat hippocampal neurons resulted in hyperpolarization of 11-14 mV and depleted action potential (AP) firing by increasing AP threshold 2- to 3-fold (Ehrengruber et al., 1997).

ABNORMAL CHANGES IN DENDRITES, SYNAPSES AND SPINES

Changes in dendrite and dendritic spine structure and morphology have been reported in almost all forms of mental retardation (Marin-Padilla, 1972; Ferrer and Gullotta, 1990; Wisniewski, 1990; Kamei et al., 1992). Within DS brain, reports indicate reduced spine number along apical dendrites of pyramidal neurons from hippocampus and cingulate gyrus (Ferrer and Gullotta, 1990). Shorter basilar dendrites, altered morphology and defective cortical layering were also found in DS infants and newborns (Takashima et al., 1981). Dendritic branching in DS infants (<6 mo) was reported to be greater than normal but declined with age so that by 2 years branching was significantly less than in normal children (Becker et al., 1986). Neuronal number may be normal during gestation but markers for dendritic spines and synapses are significantly reduced in brains from DS fetuses (Weitzdoerfer et al., 2001). Cortical lamination is also delayed and disorganized in DS individuals and may indicate disruption in axonal and dendritic connectional and functional units (Golden and Hyman, 1994).

Similar dendritic malformations have been identified in Ts65Dn mice. Ts65Dn hippocampi show significantly less synapse to neuron ratio in DG, CA3 and CA1 compared to diploid controls. This deficit was shown to be predominantly due to decreases in asymmetric (presumably excitatory) synapses in all hippocampal regions (Ayberk Kurt et al., 2004). Likewise, the temporal cortex of Ts65Dn mice was shown to have 30% fewer asymmetric synapses but no difference in symmetric synapses (Kurt et al., 2000). Layer III pyramidal neurons from Ts65Dn cortex are smaller, less branched and demonstrate a marked reduction in spine density (24%) than diploid neurons (Dierssen et al., 2003). Deficits in spine density also have been confirmed in other brain regions including fascia dentata, motor, somatosensory and entorhinal cortices (Belichenko et al., 2004). Interestingly, significant increase in spine density of basal CA1 dendrites from 6 month old Ts65Dn mice compared to diploid controls (Belichenko et al., 2004).

Both pre- and postsynaptic elements were significantly enlarged throughout Ts65Dn brain (Belichenko et al., 2004). In Ts65Dn hippocampus, there was an increase in area of synaptophysin signal, a marker for presynaptic terminals, by 145%, 139% and 131% in the fascia dentata, CA3 and CA1 regions, respectively. Specifically, within the fascia dentata and layers II-III of cortex there were significant decreases in the number of smaller (0.24-2.4 μm^2) synaptophysin positive puncta and significant increases in puncta >4.8 μm^2. Postsynaptic spines were similarly enlarged to the extent that >10% were larger than 0.5 μm^2 (where no spines larger than 0.5 μm^2 were found in diploid neurons) and many showed large irregular shapes, large vacuoles and laminar bodies. No changes were identified in dendritic shaft size. Synaptic apposition zone length (zone where pre-

and post-synaptic membrane is in direct apposition) was significantly greater in asymmetric (presumably excitatory) synapses of Ts65Dn CA1 (Ayberk Kurt et al., 2004). There also appears to be a shift in the distribution of inhibitory synapses in Ts65Dn fascia dentata. Inhibitory contacts localized at spine heads remained unchanged, but inhibitory contacts onto dendritic shafts was decreased with a equivalent increase at spine necks by ~17% (Belichenko et al., 2004). In a separate trisomy mouse model, Ts2Cje (genetically matches the Ts1Cje segment), similar dendritic anomalies were identified. Decreases in spine density and enlarged dendritic spine were found in dentate granule cells compared to diploid controls (Villar et al., 2005). This suggests that these abnormalities are not restricted merely to the Ts65Dn mouse but may be a common feature of these mouse models. Analysis of synapse architecture in other trisomy mouse models, such as Ts1Cje and Ts1Rhr will help clarify the genetic contributions to dendritic anomalies in DS.

GENETIC CONTRIBUTIONS TO SPINE DYSGENESIS

Several other genes located on Chr. 21 could be involved in DS abnormalities, as many of genes on the triplicated segment play roles in neuronal activity and dendritic spine morphology (see Galdzicki and Siarey 2003}. We will focus on two candidate genes Tiam1 and Dscr1.

The Rac1 specific guanine nucleotide exchange factor (GTPase), Tiam1, illustrates how disruptions in signaling could mediate abnormal synaptic morphology and plasticity. Rac1 and other GTPases modulate spine morphology and regulate actin cytoskeletal elements involved in plasticity mechanisms (Newey et al., 2005). Since Tiam1 activates Rac1 through its GTPase activity an overexpression of Tiam1 would

raise Rac1 activity levels. Overexpression of constitutively activated Rac1 leads to enlarged spine heads (Tashiro and Yuste, 2004) and also induced the clustering of AMPA receptors and increased the amplitude of miniature EPSCs (Wiens et al., 2005). The NMDA receptor plays an important role in the activity-dependent structural remodeling of dendrites by activation Tiam1 (Tolias et al., 2005). Tiam1 is phosphorylated by CAMKII (Fleming et al., 1999), a kinase crucial for spatial memory and hippocampal synaptic plasticity (Silva, 2003). Furthermore, phosphatidylinositol-3,4,5-trisphosphate binds to Tiam1 promoting Tiam1 membrane localization (Sander et al., 1998), hence implicating PI3K and its downstream signaling cascades. Therefore overexpression of Tiam1 in Ts65Dn mice may be important to the disruptions in synaptic plasticity mechanisms as well as contributing to the structural abnormalities in dendritic spines. Tiam1 may directly account for the increased size of spine heads seen in Ts65Dn mice and indirectly the reduction in dendritic spines. If spines are abnormally large to start with, because of too much Rac1 activation, compensatory mechanisms may reduce the number of spines. Nevertheless, Tiam1 gene is not located on the Ts1Cje segment and therefore deficits in synaptic plasticity, spine density and spine malformations cannot be a result of its overexpression in Ts1Cje (Ts2Cje) mice.

Another Chr. 21 gene, DSCR1, is implicated in disrupted neuronal functioning. A peptide fragment of DSCR1, calcipressin 1, directly binds and inhibits the phosphatase calcineurin (Chan et al., 2005), a protein involved in the regulation of transcription factors. The DSCR1 gene is suggested to have a role in learning defects in the Drosophila that is attributed to biochemical perturbations (Chang et al., 2003). The Drosophila homolog of DSCR1 specifically affects the mitochondrial functioning, in that it regulates

the activity of the ADP/ATP translocator (Chang and Min, 2005). Mitochondria are localized in high density at synapses and their functioning is critical to dendritic spine formation and synaptic plasticity (Li et al., 2004). Thus DSCR1 may influence neuronal functioning through both mitochondrial activity and calcineurin phosphatase signaling cascades.

On human chromosome 21 there are also genes not orthologous to mouse chromosome 16 that have been suggested to contribute to DS neurological phenotypes. Calcium-binding protein S100B (localized to mouse chromosome 10, Figure 1) have been found to exert a spectrum of neurodegenerative activities with potential impact on neuronal cytoskeleton and have been suggested to play role in DS and AD (Shapiro et al., 2004). New mouse models that carry triplication of fragments of mouse chromosome 10 and 17 fragments fragment that are orthologous to human chromosome 21 with and without Ts65Dn fragment are needed to address their role in DS (Vacik et al., 2005).

SYNAPSE DYSGENESIS CORRELATED TO ELECTROPHYSIOLOGICAL DATA

All reports examining synaptic plasticity in Ts65Dn, Ts1Cje and Tc1 indicate that basal excitatory stimulus response is normal (Siarey et al., 1997; Siarey et al., 1999; Kleschevnikov et al., 2004; Costa and Grybko, 2005; O'Doherty et al., 2005; Siarey et al., 2005). This is surprising considering the abnormal spine density and increases in the size of pre- and postsynaptic elements. Compensatory changes may be involved that establish normal synaptic activity of integrated signals. The relative decreases in excitatory synapses may be compensated by redistribution of inhibitory inputs or changes in

Figure 3

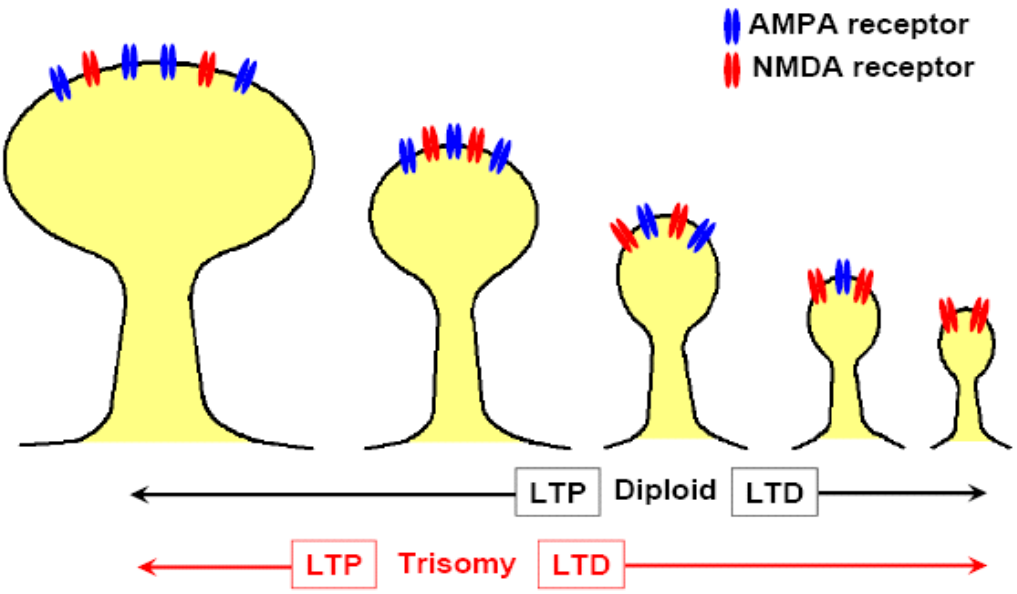

Figure 3. Hypothetical model relating dendritic spine size to probabilities of potentiation and depression of synapses. Baseline for synapses in diploid/control mice are represented as medium sized spines prior to synaptic activation by tetanus that can be enlarged (LTP) or diminished (LTD) with equal magnitude. Spines from trisomy mice are, under basal conditions, enlarged already and therefore the opportunity to undergo further enhancement is diminished. By contrast enlarged spines have an enhanced ability to undergo LTD due to the potential for greater size reduction. This model does not take into account the reduction in the length of spine neck that was reported in Ts65Dn dentate gyrus neurons (Belichenko et al., 2004).

glutamatergic receptor number and/or distribution. More sensitive experiments are needed to determine whether changes in spine morphology and density correlate with changes in basal activity at the single neuron level. Failures analysis or examining spontaneous events under whole-cell recordings as Kleschevnikov et al., (2004), who showed there to be an increased frequency of mIPSCs in the Ts65Dn dentate gyrus, are warranted. The changes in synapse dysgenesis are consistent with results of synaptic plasticity paradigms. Large dendritic spines are considered less plastic and permanent fixtures of neuronal transmission, whereas smaller spines are more immature and are

amenable to plastic changes. Protocols that induce LTP have been shown to correlate with formation of new spines and enlargement of existing ones (Matsuzaki et al., 2004). Conversely LTD has been associated with decreases in spine size of hippocampal synapses (Zhou et al., 2004; Tada and Sheng, 2005). As mentioned above, LTP is decreased and LTD is enhanced in Ts65Dn and Ts1Cje hippocampus. If spines from these DS mouse models are enlarged under basal condition, then such may preclude potentiation of stimulated pathways, such that potentiation has reached plateau. Similarly the enhanced LTD may be explained in that the enlarged spine heads are better able to diminish in size compared to smaller spine heads found in diploid mice (Figure 3).

SUMMARY

The mouse models of DS provide a powerful tool to elucidate the mechanisms underlying suboptimal neural functioning in Down syndrome individuals at the neuronal level and then affecting simple and complex neuronal networks. We hypothesize that overexpression of genes from chromosome 21 shifts biological homeostasis in the Down syndrome brain to a new less functional state. In this altered steady state mechanisms of development, structure and plasticity malfunction due to compromises caused by the neurophysiological impact of overexpressed genes from trisomy 21.

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